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(54) Title: METHODS AND PRODUCTS FOR IDENTIFICATION AND ASSESSMENT OF TLR LIGANDS

STIMULATION RPMI 8226 (FACS)
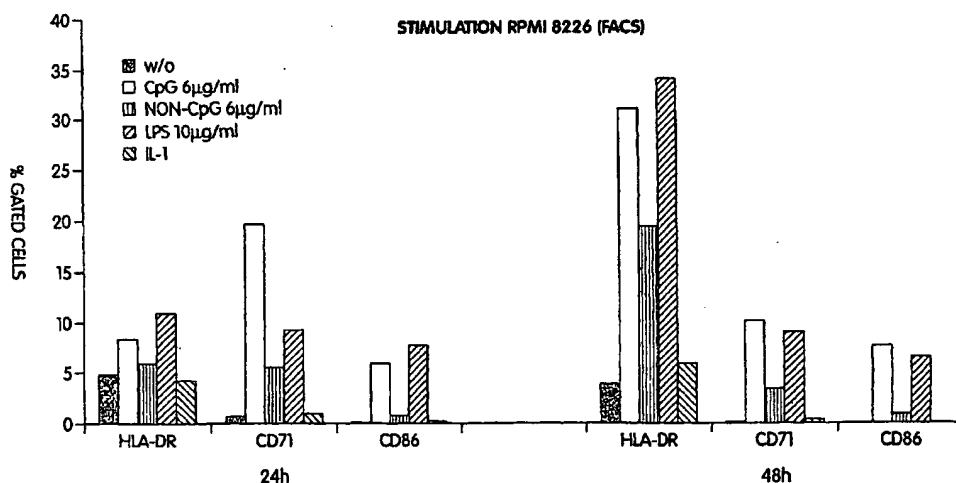
(57) Abstract: The invention provides in part novel screening methods and compositions for identifying and distinguishing between candidate immunomodulatory compounds. The invention further provides methods for assessing biological activity of composition containing a known TLR ligand. These latter methods can be used for quality assessment and selection of various lots of test compositions, including pharmaceutical products for clinical use.

GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW),
Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), Euro-
pean (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR,
GB, GR, HU, IE, IT, LU, MC, NL, PL, PT, RO, SE, SI, SK,
TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW,
ML, MR, NE, SN, TD, TG).

METHODS AND PRODUCTS FOR IDENTIFICATION AND ASSESSMENT
OF TLR LIGANDS

Background of the Invention

5   Nucleic acids with immunostimulatory activity have been identified. The first
recognized immunostimulatory motif was the CpG motif in which at least the C of the
dinucleotide was unmethylated. It has been postulated that mammalian subjects recognize the
unmethylated dinucleotide as being of bacterial origin, and thus mount a heightened immune
response following exposure. The ensuing immune response includes both cell mediated and
10   humoral aspects. Since the discovery of the CpG immunostimulatory motif, other
immunostimulatory motifs have also been identified including the poly-T and T-rich motifs,
the TG motif and the poly-G motif. In some instances, immunostimulation has also been
observed in response to exposure to methylated CpG motifs and motif-less nucleic acids
having phosphorothioate backbone linkages.

15   The responses induced by immunostimulatory nucleic acids are varied and can include
production and secretion of cytokines, chemokines, and other growth factors. The nucleic
acids can induce a heightened immune stimulation regardless of whether an antigen is also
introduced to the subject. Identification of new motifs as well as of subtle differences
between response profiles of different nucleic acids oftentimes can be laborious, and a high
20   throughput system for screening nucleic acids for their ability to be immunostimulatory as
well as to determine the profile of responses they induce would be useful.

Summary of the Invention

          The invention provides in its broadest sense screening methods and tools for
25   identification and discrimination of immunomodulatory molecules and assessment and
standardization of samples containing known immunomodulatory molecules. The
immunomodulatory molecules can be immunostimulatory or immunoinhibitory, and most
preferably are Toll-like receptor (TLR) ligands.

          In one aspect, the invention provides a screening method for identifying TLR agonists.
30   The method comprises contacting a cell line endogenously expressing at least one TLR with a
test compound and measuring a test level of TLR signaling activity, wherein a positive test
level is indicative of a TLR agonist (i.e., an immunostimulatory compound). The positive test

level may be apparent without referring to a control. Preferably, however, it is determined relative to a control (i.e., the TLR signaling activity from a reference compound).

In some embodiments, the reference compound is a compound that induces no response (i.e., a zero response) or a minimal response. In this case, a test level that is greater

5   than the reference level is indicative of a compound with TLR signaling activity. More preferably, the reference compound is a compound that induces a positive response (i.e., a non-zero response) and that is immunostimulatory. These reference compounds are referred to herein as negative and positive reference compounds, respectively. If the reference compound is immunostimulatory (i.e., a positive reference compound), a non-zero test level

10·   that is lower than the reference level is still indicative of an immunostimulatory test compound. In this latter embodiment, the test compound is less immunostimulatory than the reference compound (for that particular readout), but it is nonetheless immunostimulatory given the non-zero response induced. There may be one or more concurrent or consecutive assays with a negative reference compound, a positive reference compound, or both. The

15   reference may also be a standard curve or data generated previously.

In a related aspect, the screening method involves exposing the same cell to a positive reference compound and a test compound in order to identify a test compound that inhibits the immunostimulatory response of the positive reference compound (i.e., a TLR antagonist or an immunoinhibitory compound).

20   In still a related aspect, the screening method involves exposing the same cells to a positive reference compound and a test compound in order to identify a test compound that enhances the immunostimulatory response of the positive reference compound (i.e., an enhancer).

In both of these latter aspects, the assay requires a co-incubation of the positive

25   reference compound, the test compound and the cells. Separate assays with positive reference compound alone and optionally negative reference compound alone are usually also performed.

The positive reference compound is a known TLR ligand. Non-limiting examples include but are not limited to TLR3 ligands, TLR7 ligands, TLR8 ligands and TLR9 ligands.

30   In some embodiments, the positive reference compound is an immunostimulatory nucleic acid. In some embodiments, the positive reference compound is a CpG nucleic acid, a poly-T nucleic acid, a T-rich nucleic acid or a poly-G nucleic acid. Another example of a positive

reference compound is a nucleic acid comprising a backbone that contains at least one phosphorothioate linkage.

It has been further discovered according to the invention that the RPMI 8226 cell line expresses TLR7 and responds to the imidazoquinoline compound R-848 (Resiquimod) which

5   is known to signal through TLR7 and TLR8. Accordingly, the screening method can be performed using RPMI 8226, Raji or RAMOS cells and an imidazoquinoline compound such as R-848 or R-847 (Imiquimod) as the positive reference compound.

In one embodiment, the test compound is a nucleic acid such as but not limited to a DNA, an RNA and a DNA/RNA hybrid. The test compound may be a nucleic acid that does

10   not comprise motif selected from the group consisting of a CpG motif, a poly-T motif, a T-rich motif and a poly-G motif. The test compound may be a nucleic acid that comprises a phosphorothioate backbone linkage. In another embodiment, the test compound is a non-nucleic acid small molecule. The non-nucleic acid small molecule may be derived from a molecular library. In other embodiments, the test compound comprises amino acids,

15   carbohydrates such as polysaccharides. It may be a hormone or a lipid or contain moieties derived therefrom. In other embodiments, the test compounds are putative ligands for TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, TLR10 or TLR11.

In one embodiment, the cell is a RPMI 8226 cell, a Raji cell, a RAMOS cell, a THP-1 cells, a Nalm cell or a KG-1 cell and the TLR is TLR9. In another embodiment, the cell is a

20   RPMI 8226 cell, a Raji cell or a RAMOS cell and the TLR is TLR7. In yet another embodiment, the cell is a KG-1 cell, a Nalm cell, a Raji cell, a RAMOS cell, a Jurkat cell, a Hela cell, a Hep-2 cell, a Hep-2 cells, a A549 cell, a Bewo cell, an NK-92 cell or an NK-92 MI cell and the TLR is TLR3.

In another embodiment, the cell is an RPMI 8226 cell and the TLR is TLR7 or TLR9.

25   In still another embodiment, the cell is a Raji cell and the TLR is TLR9, TLR7 or TLR3.

Depending upon the embodiment, the TLR signaling activity may be measured or detected in a number of ways. In one embodiment, the TLR signaling activity is measured by cytokine, chemokine, or growth factor secretion. The cytokine secretion may be selected from the group consisting of IL-6 secretion, IL-10 secretion, IL-12 secretion, IFN-α secretion

30   and TNF-α secretion, but is not so limited. The chemokine secretion may be IP-10 secretion or IL-8 secretion, but is not so limited.

In another embodiment, the TLR signaling activity is measured by antibody secretion. The antibody secretion may be IgM secretion, but is not limited to this antibody subtype.

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In another embodiment, the TLR signaling activity is measured by phosphorylation. The total level of phosphorylation in the cell or the level of phosphorylation of particular factors in the cell may be measured. These factors are preferably signaling factors and can be selected from the group consisting of IRAK, ERK, MyD88, TRAF6, p38, Jun, c-fos, and

5 subunits of NF-κB, but are not so limited.

In still a further embodiment, the TLR signaling activity is measured by cell surface marker expression. In one embodiment, the TLR signaling activity is measured by an increase in cell surface marker expression. Examples of cell surface markers to be analyzed include CD71, CD86, HLA-DR, CD80, HLA Class I, CD54 and CD69. In other

10 embodiments, the TLR signaling activity is measured by a decrease in cell surface marker expression. Cell surface marker expression can be determined using flow cytometry. TLR signaling activity can also be measured by protein production (e.g., by Western blot).

In another embodiment, the TLR signaling activity is measured by gene expression. Gene expression profiles may be determined using Northern blot analysis or RT-PCR that

15 uses mRNA or total RNA as a starting material. The gene expression of interest may be that of the chemokines and cytokines and cell surface molecules recited above. Gene expression analysis can be performed using microarray techniques.

In yet another embodiment, the TLR signaling activity is measured by cell proliferation. Cell proliferation assays can be measured in a number of ways including but

20 not limited to ^{3}H-thymidine incorporation.

In one embodiment, the cell is an RPMI 8226 cell and TLR signaling is indicated by expression of a marker such as CD71, CD86 and/or HLA-DR or by expression, production or secretion of a factor such as IL-8, IL-10, IP-10 and/or TNF-α. Preferably, in this latter embodiment, the RPMI 8226 cell is unmodified. In another embodiment, the cell is a Raji

25 cell and the TLR signaling is indicated by IL-6 or IFN-α2 expression, production or secretion. In yet another embodiment, the cell is a RAMOS cell and the TLR signaling is indicated by CD80 cell surface expression.

TLR signaling activity can be measured via a native readout or an artificial readout or both. A native readout is one that does not rely on introduction of a reporter construct into the

30 cell of interest.

The cell line may be used in a modified or unmodified form. In one embodiment, the cell line is transfected with a reporter construct. The transfection may be transient or stable. The reporter construct generally comprises a promoter, a coding sequence and a

polyadenylation signal. The coding sequence may comprise a reporter sequence selected from the group consisting of an enzyme (e.g., luciferase, alkaline phosphatase, β-galactosidase, chloramphenicol acetyltransferase (CAT), secreted alkaline phosphatase, etc.), a bioluminescence marker (e.g., green fluorescent protein (GFP, U.S. Patent No. 5,491,084),

5   etc.), a surface-expressed molecule (e.g., CD25), a secreted molecule (e.g., IL-8, IL-12 p40, TNF-α, etc.), and other detectable protein sequences known to those of skill in the art. Preferably, the coding sequence encodes a protein, the level or activity of which can be quantified, with preferably a wide linear range.

In some embodiments, the promoter is a promoter that is responsive to TLR signaling

10   pathways (i.e., a "TLR responsive promoter"). In some embodiments, the promoter contains a binding site for a transcription factor activated upon CpG nucleic acid exposure, such as for example NF-κB. In other embodiments, the promoter contains a binding site for a transcription factor that is activated by a positive reference compound other than CpG nucleic acids. The transcription factor binding site may be selected from the group consisting of a

15   NF-κB binding site, an AP-1 binding site, a CRE, a SRE, an ISRE, a GAS, an ATF2 binding site, an IRF3 binding site, an IRF7 binding site, an NFAT binding site, a p53 binding site, an SRF binding site, and a TARE, as well as others known to those of skill in the art.

In another embodiment, the promoter contains a functional promoter element from an IL-1 gene, an IL-6 gene, an IL-8 gene, an IL-10 gene, an IL-12 p40 gene, an IFN-α1 gene, an

20   IFN-α4 gene, an IFN-β gene, an IFN-γ gene, a TNF-α gene, a TNF-β gene, an IP-9 gene, an IP-10 gene, a RANTES gene, an ITAC gene, a MCP-1 gene, an IGFBP4 gene, a CD54 gene, a CD69 gene, a CD71 gene, a CD80 gene, a CD86 gene, a HLA-DR gene, and a HLA class I gene.

The TLR responsive promoter may be a TLR1 responsive promoter, a TLR2

25   responsive promoter, a TLR3 responsive promoter, a TLR4 responsive promoter, a TLR5 responsive promoter, a TLR6 responsive promoter, a TLR7 responsive promoter, a TLR8 responsive promoter, a TLR9 responsive promoter, a TLR10 responsive promoter or a TLR11 responsive promoter.

In these latter embodiments, the cell line may be transfected with a reporter construct

30   having a promoter derived from a particular cytokine, chemokine, or cell surface marker, and a unique reporter coding sequence conjugated thereto. In this way, the readout from a particular reporter construct is a surrogate readout for cytokine, chemokine, or cell surface marker readout. Measuring readout from the reporter coding sequences described herein is in

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some instances easier than measuring cytokine or chemokine secretion, or upregulation of a cell surface marker.

In these latter embodiments, the cell line may be transfected with a number of reporter constructs each having a promoter derived from a particular cytokine, chemokine, or cell

5   surface marker, and a unique distinguishable coding sequence conjugated thereto. In these embodiments, multiple readouts are possible from one screen. In other embodiments, multiple native readouts are also possible from one screen.

In a related embodiment, the cell may be further transfected with a nucleic acid that codes for a TLR polypeptide or a fragment thereof. Preferably, the TLR is one that is not

10   endogenously expressed by the cell. As an example, if the cell is an RPMI 8226 cell which has been shown to express TLR7 and TLR9 according to the invention, then it may be modified to express TLRs other than these (e.g., TLR8) in some embodiments. In this aspect, the RPMI 8226 cell is responsive to TLR8 ligands. In preferred embodiments, the TLR is a human TLR (i.e., hTLR).

15   In another aspect, the invention provides an RPMI 8226 cell transfected with a TLR nucleic acid. In still another embodiment, the TLR nucleic acid is selected from the group consisting of TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR8, TLR10 and TLR11. The encoded TLRs nucleic acids can derive from human or non-human sources. Examples of non-human sources include, but are not limited to, murine, bovine, canine, feline, ovine,

20   porcine, and equine species. Other species include chicken and fish, e.g., aquaculture species. The TLR nucleic acids can also include chimeric sequences consisting of domains originating from different species. In preferred embodiments, the TLR is a human TLR.

In still another aspect, the invention provides kits including the cells lines (e.g., the RPMI 8226 cell line), the reporter constructs and/or expression constructs described above,

25   and instructions for use.

Other aspects of the invention provide methods for analyzing the biological activity of individual lots of material containing previously identified specific TLR ligands (i.e., specific compounds which are ligands for a particular TLR) intended for use as, or for use in the preparation of, pharmaceutical compositions. The methods permit a qualitative and,

30   importantly, a quantitative assessment of biological activity of individual lots of TLR ligands, pre-formulation as well as post-formulation. Such methods are useful in the manufacture and validation of pharmaceutical compositions containing, as an active agent, at least one specific ligand of at least one specific TLR. The specific TLR can be any known TLR, including

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without limitation TLR3, TLR7, TLR8 and TLR9. The specific TLR ligand is an isolated
TLR ligand, either found in nature or synthetic (not found in nature), including in particular
certain nucleic acid molecules and small molecules. Nucleic acid molecules that are specific
TLR ligands include synthetic and naturally-occurring oligonucleotides having specific base

5   sequence motifs. Furthermore, specific TLR ligands include both agonists and antagonists of
specific TLR.

        These methods are to be distinguished from test procedures and acceptance criteria for
new drug substances and new drug products which are classified as chemical substances.
Unlike the afore-mentioned test procedures and acceptance criteria, the methods of the instant

10   invention deal specifically with characterizing drug substances and drug products which are
classified as oligonucleotides. Oligonucleotides are explicitly excluded in ICH Topic Q6A
Specifications: Test Procedures and Acceptance Criteria for New Drug Substances and New
Drug Products: Chemical Substances, Step 4 – Consensus Guideline: 6 October 1999, § 1.3.

        Further still, the methods of the instant invention are to be distinguished from test

15   procedures and acceptance criteria for biotechnological/biological products. Unlike the afore-
mentioned test procedures and acceptance criteria, the methods of the invention deal
specifically with characterizing biotechnological/biological products which are classified as
DNA products. DNA products are explicitly excluded in ICH Harmonised Tripartite
Guideline Specifications: Test Procedures and Acceptance Criteria for

20   Biotechnological/Biological Products, Step 4 – 10 March 1999, § 1.3.

        In one aspect, the invention provides a method for quality assessment of a test
composition containing a known TLR ligand. The method according to this aspect of the
invention involves measuring a reference activity of a reference composition comprising a
known TLR ligand, wherein the known TLR ligand is a nucleic acid molecule; measuring a

25   test activity of a test composition comprising the known TLR ligand; and comparing the test
activity to the reference activity. In one embodiment the method further involves the step of
selecting the test composition if the test activity falls within a predetermined range of variance
about the reference activity.

        In one embodiment, the reference composition is a first production lot of a

30   pharmaceutical composition comprising the known TLR ligand, and the test composition is a
second production lot of a pharmaceutical composition comprising the known TLR ligand.
This embodiment is particularly useful as a method for developing and applying acceptance
criteria for finished pharmaceutical products containing a known TLR ligand.

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In another embodiment, the reference composition is a first in-process lot of a composition comprising the known TLR ligand, and the test composition is a second in-process lot of a composition comprising the known TLR ligand. This embodiment is particularly useful as a method for developing and applying acceptance criteria for raw

5   materials and/or other in-process materials containing a known TLR ligand bound for use in a pharmaceutical product.

In one embodiment according to this aspect of the invention, measuring the reference activity involves contacting the reference composition with an isolated cell expressing a TLR responsive to the known TLR ligand, and measuring the test activity involves contacting the

10 test composition with the isolated cell expressing the TLR responsive to the known TLR ligand. Further, in one embodiment the isolated cell expressing the TLR responsive to the known TLR ligand includes an expression vector for the TLR responsive to the known TLR ligand. Such expression vector, and likewise for any expression vector according to the instant invention, can be introduced into the cell using any suitable method.

15 In one embodiment, the isolated cell expressing the TLR responsive to the known TLR ligand naturally expresses the TLR responsive to the known TLR ligand. Such a cell can be naturally occurring or it can be a cell line, provided the cell does not include an expression vector introduced into the cell for the purpose of artificially inducing the cell to express or overexpress the TLR.

20 In one particular embodiment, the isolated cell expressing the TLR responsive to the known TLR ligand is RPMI 8226. In another embodiment, the isolated cell expressing the TLR responsive to the known TLR ligand is Raji, RAMOS, Nalm, THP-1 or KG-1 and the TLR is TLR9. In another embodiment, the isolated cell expressing the TLR responsive to the known TLR ligand is RPMI 8226, Raji or RAMOS and the TLR is TLR7. In yet another

25 embodiment, the isolated cell expressing the TLR responsive to the known TLR ligand is a KG-1 cell, a Nalm cell, a Raji cell, a RAMOS cell, a Jurkat cell, a Hela cell, a Hep-2 cell, a Hep-2 cells, a A549 cell, a Bewo cell, an NK-92 cell or an NK-92 MI cell and the TLR is TLR3.

Further according to this aspect of the invention, in one embodiment measuring the

30 reference activity and measuring the test activity each comprises measuring signaling activity mediated by a TLR responsive to the known TLR ligand. As described in greater detail elsewhere herein, TLR signaling involves a series of intracellular signaling events. These signaling events give rise to various downstream products, including certain transcription

factors (e.g., NF-κB and AP-1), cytokines, chemokines, etc., which can affect the activity of certain gene promoters. For example, in one embodiment the signaling activity is activity of a reporter gene or reporter construct under the control of a NF-κB response element.

In other embodiments, the signaling activity is activity of a reporter gene or reporter

5   construct under the control of an interferon-stimulated response element (ISRE); an IFN-α promoter; an IFN-β promoter; an IL-6 promoter; an IL-8 promoter; an IL-12 p40 promoter; a RANTES promoter; an IL-10 promoter or an IP-10 promoter.

In one embodiment, the known TLR ligand is an immunostimulatory nucleic acid. An immunostimulatory nucleic acid can include, without limitation, a CpG nucleic acid. In

10   another embodiment, the known TLR ligand is an immunoinhibitory nucleic acid. When the known TLR ligand is a TLR antagonist (e.g., an immunoinhibitory oligonucleotide), the method according to this aspect of the invention can further involve measuring the reference activity of the reference composition and measuring the test activity of the test composition, each performed in the presence of a known immunostimulatory TLR ligand.

15   In various embodiments, the known TLR ligand is a ligand for a particular TLR. Thus in one embodiment the known TLR ligand is a TLR9 ligand. More specifically, in one embodiment the known TLR ligand is a CpG nucleic acid.

In one embodiment, the known TLR ligand is a TLR3 ligand. Such a ligand can include, for example, a double-stranded RNA or a homolog thereof.

20   In one embodiment, the known TLR ligand is a TLR7 ligand. In one embodiment the known TLR ligand is a TLR8 ligand.

The invention provides in another aspect a method for quality assessment of a test lot of a pharmaceutical product containing a known TLR9 ligand. The method according to this aspect of the invention involves measuring a reference activity of a reference lot of a

25   pharmaceutical product comprising a known TLR9 ligand, wherein the known TLR9 ligand is a nucleic acid molecule; measuring a test activity of a test lot of a pharmaceutical product comprising the known TLR9 ligand; comparing the test activity to the reference activity; and rejecting the test lot if the test activity falls outside of a predetermined range of variance about the reference activity.

30   In one embodiment according to this aspect of the invention, the known TLR9 ligand is an oligonucleotide having a base sequence provided by 5'-TCG TCG TTT TGT CGT TTT GTC GTT-3' (SEQ ID NO:1).

In one embodiment according to this aspect of the invention, the known TLR9 ligand is an oligonucleotide having a base sequence provided by 5'-TCG TCG TTT TGA CGT TTT GTC GTT-3' (SEQ ID NO:139).

In one embodiment according to this aspect of the invention, the known TLR9 ligand is an oligonucleotide having a base sequence provided by 5'-TCG TCG TTT TGT CGT TTT TTT CGA-3' (SEQ ID NO:140).

In one embodiment according to this aspect of the invention, the known TLR9 ligand is an oligonucleotide having a base sequence provided by 5'-TCG TCG TTT CGT CGT TTC GTC GTT-3' (SEQ ID NO:141).

In one embodiment according to this aspect of the invention, the known TLR9 ligand is an oligonucleotide having a base sequence provided by 5'-TCG TCG TTT CGT CGT TTT GTC GTT-3' (SEQ ID NO:142).

In one embodiment according to this aspect of the invention, the known TLR9 ligand is an oligonucleotide having a base sequence provided by 5'-TCG TCG TTT TTC GGT CGT TTT-3' (SEQ ID NO:143).

In one embodiment according to this aspect of the invention, the known TLR9 ligand is an oligonucleotide having a base sequence provided by 5'-TCG TCG TTT TTC GTG CGT TTT T-3' (SEQ ID NO:144).

In one embodiment according to this aspect of the invention, the known TLR9 ligand is an oligonucleotide having a base sequence provided by 5'-TCG TCG TTT TCG GCG GCC GCC G-3' (SEQ ID NO:145).

In one embodiment according to this aspect of the invention, the known TLR9 ligand is an oligonucleotide having a base sequence provided by 5'-TCG TC_G TTT TAC_GGC GCC_GTG CCG-3' (SEQ ID NO:146), wherein every internucleoside linkage is phosphorothioate except for those indicated by "_", which are phosphodiester.

Each of the limitations of the invention can encompass various embodiments of the invention. It is, therefore, anticipated that each of the limitations of the invention involving any one element or combinations of elements can be included in each aspect of the invention.

Brief Description of the Figures

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Fig. 1 is a bar graph showing cell surface expression of various markers by RPMI 8226 24 hours and 48 hours following stimulation with CpG nucleic acid (SEQ ID NO: 1), non-CpG nucleic acid (SEQ ID NO: 2), LPS and IL-1.

Fig. 2 is a bar graph showing IL-8 production by RPMI 8226 24 hours after exposure
5   to CpG nucleic acid (SEQ ID NO: 1), non-CpG nucleic acid (SEQ ID NO: 2), R-848 and LPS.

Fig. 3 is a bar graph showing IL-6 production by RPMI 8226 24 hours after exposure to CpG nucleic acid (SEQ ID NO: 1), non-CpG nucleic acid (SEQ ID NO: 2), R-848 and LPS.

10 Fig. 4 is a bar graph showing IP-10 production by RPMI 8226 24 hours after exposure to CpG nucleic acid (SEQ ID NO: 1), non-CpG nucleic acid (SEQ ID NO: 2), R-848 and LPS.

Fig. 5 is a bar graph showing IL-10 production by RPMI 8226 24 hours after exposure to CpG nucleic acid (SEQ ID NO: 1), non-CpG nucleic acid (SEQ ID NO: 2), R-848 and
15 LPS.

Fig. 6 is a dose response curve showing fold induction of IL-8 production 24 hours after exposure to CpG nucleic acid (SEQ ID NO: 1) and non-CpG nucleic acid (SEQ ID NO: 2). The EC_{50} for CpG nucleic acid is 19 nM and the EC_{50} for non-CpG nucleic acid is 263 nM.

20 Fig. 7 is a bar graph showing NF-κB activation in RPMI 8226 transfected transiently with a NF-κB-luciferase reporter gene construct as a function of cell density and nucleic acid amount transfected, following exposure to CpG nucleic acid (SEQ ID NO: 1), LPS and TNF-α. NF-κB activation is measured by luciferase activity.

Fig. 8 is a bar graph showing RT-PCR results from RNA isolated from RPMI 8226
25 using gene specific primers for TLR7, TLR8 and TLR9 genes.

Fig. 9 is a dose response curve showing IP-10 production induced by SEQ ID NO: 1, and inhibition thereof in the presence of SEQ ID NO: 151, a immunoinhibitory nucleic acid.

Fig. 10 is a bar graph showing the results of a TLR9 RT-PCR analysis of a number of cell lines.

30 Fig. 11 is a bar graph showing the results of a TLR7 RT-PCR analysis of a number of cell lines.

Fig. 12 is a bar graph showing the results of a TLR3 RT-PCR analysis of a number of cell lines.

Fig. 13 is a bar graph showing the results of a TLR3, TLR7, TLR8 and TLR9 RT-PCR analysis of the Raji cell line.

Fig. 14 is a graph showing IL-6 production by the Raji cell line upon stimulation with various ODN (SEQ ID NO:1; SEQ ID NO:154; SEQ ID NO:158; SEQ ID NO:160; SEQ ID NO:159; SEQ ID NO:161).

Fig. 15 is a bar graph showing IL-6 production of the Raji cell line upon stimulation with poly I:C and R-848.

Fig. 16 is a bar graph showing IFN-α2 production by the Raji cell line upon stimulation with CpG ODN (SEQ ID NO: 1), R-848 and poly I:C.

Fig. 17 is a bar graph showing CD80 expression (by flow cytometry) by the RAMOS cell line upon stimulation with CpG ODN (SEQ ID NO: 1) and non-CpG ODN (SEQ ID NO: 2).

Fig. 18A is a bar graph showing the induction of NF-κB by 293 fibroblast cells transfected with human TLR9 in response to exposure to various stimuli, including CpG-ODN, GpC-ODN, LPS, and medium.

Fig. 18B is a bar graph showing the amount of IL-8 produced by 293 fibroblast cells transfected with human TLR9 in response to exposure to various stimuli, including CpG-ODN, GpC-ODN, LPS, and medium.

Fig. 19 is a bar graph showing the induction of NF-κB-luc produced by stably transfected 293-mTLR9 cells in response to exposure to various stimuli, including CpG-ODN, methylated CpG-ODN (Me-CpG-ODN), GpC-ODN, LPS and medium.

Fig. 20 is a bar graph showing the induction of NF-κB-luc produced by stably transfected 293-hTLR9 cells in response to exposure to various stimuli, including CpG-ODN, methylated CpG-ODN (Me-CpG-ODN), GpC-ODN, LPS and medium.

Fig. 21 is a series of gel images depicting the results of reverse transcriptase-polymerase chain reaction (RT-PCR) assays for murine TLR9 (mTLR9), human TLR9 (hTLR9), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in untransfected control 293 cells, 293 cells transfected with mTLR9 (293-mTLR9), and 293 cells transfected with hTLR9 (293-hTLR9).

It is to be understood that the Figures are not required for enablement of the invention.

Brief Description of Sequences

SEQ ID NO:1 is the nucleotide sequence of an immunostimulatory nucleic acid (TLR9 ligand).

SEQ ID NO:2 is the nucleotide sequence of a non-CpG nucleic acid.

SEQ ID NO:3 is the nucleotide sequence of human TLR2 cDNA (U88878).

5   SEQ ID NO:4 is the amino acid sequence of human TLR2 protein (AAC34133).

SEQ ID NO:5 is the nucleotide sequence of murine TLR2 cDNA (AF165189).

SEQ ID NO:6 is the amino acid sequence of murine TLR2 protein (NP_036035).

SEQ ID NO:7 is the nucleotide sequence of human TLR3 cDNA (NM_003265).

SEQ ID NO:8 is the amino acid sequence of human TLR3 protein (NP_003256).

10   SEQ ID NO:9 is the nucleotide sequence of murine TLR3 cDNA (AF355152).

SEQ ID NO:10 is the amino acid sequence of murine TLR3 protein (AAK26117).

SEQ ID NO:11 is the nucleotide sequence of human TLR4 cDNA (U88880).

SEQ ID NO:12 is the nucleotide sequence of human TLR4 cDNA transcript variant 4 (NM_138557).

15   SEQ ID NO:13 is the nucleotide sequence of human TLR4 cDNA transcript variant 2 (NM_138556).

SEQ ID NO:14 is the nucleotide sequence of human TLR4 cDNA transcript variant 1 (NM_138554).

SEQ ID NO:15 is the nucleotide sequence of human TLR4 cDNA transcript variant 3 20   (NM_003266).

SEQ ID NO:16 is the amino acid sequence of human TLR4 protein isoform A (NP_612564).

SEQ ID NO:17 is the amino acid sequence of human TLR4 protein isoform B (NP_612566).

25   SEQ ID NO:18 is the amino acid sequence of human TLR4 protein isoform C (NP_003257).

SEQ ID NO:19 is the amino acid sequence of human TLR4 protein isoform D (NP_612567).

SEQ ID NO:20 is the nucleotide sequence of murine TLR4 cDNA (NM_021297).

30   SEQ ID NO:21 is the nucleotide sequence of murine TLR4 mRNA (AF185285).

SEQ ID NO:22 is the nucleotide sequence of murine TLR4 mRNA (AF110133).

SEQ ID NO:23 is the amino acid sequence of murine TLR4 protein (AAD29272).

SEQ ID NO:24 is the amino acid sequence of murine TLR4 protein (AAF04278).

SEQ ID NO:25 is the nucleotide sequence of human TLR5 cDNA (AB060695).

SEQ ID NO:26 is the amino acid sequence of human TLR5 protein (BAB43558).

SEQ ID NO:27 is the amino acid sequence of human TLR5 protein (O60602).

SEQ ID NO:28 is the amino acid sequence of human TLR5 protein (AAC34136).

SEQ ID NO:29 is the nucleotide sequence of murine TLR5 cDNA (AF186107).

SEQ ID NO:30 is the amino acid sequence of murine TLR5 protein (AAF65625).

SEQ ID NO:31 is the nucleotide sequence of human TLR7 cDNA (AF240467).

SEQ ID NO:32 is the nucleotide sequence of human TLR7 cDNA (AF245702).

SEQ ID NO:33 is the nucleotide sequence of human TLR7 cDNA (NM_016562).

SEQ ID NO:34 is the amino acid sequence of human TLR7 protein (AAF60188).

SEQ ID NO:35 is the amino acid sequence of human TLR7 protein (AAF78035).

SEQ ID NO:36 is the amino acid sequence of human TLR7 protein (NP_057646).

SEQ ID NO:37 is the amino acid sequence of human TLR7 protein (Q9NYK1).

SEQ ID NO:38 is the nucleotide sequence of murine TLR7 cDNA (AY035889).

SEQ ID NO:39 is the nucleotide sequence of murine TLR7 splice variant (NM_133211).

SEQ ID NO:40 is the nucleotide sequence of murine TLR7 splice variant (AF334942).

SEQ ID NO:41 is the amino acid sequence of murine TLR7 protein (AAK62676).

SEQ ID NO:42 is the amino acid sequence of murine TLR7 protein (AAL73191).

SEQ ID NO:43 is the amino acid sequence of murine TLR7 protein (AAL73192).

SEQ ID NO:44 is the amino acid sequence of murine TLR7 protein (NP_573474).

SEQ ID NO:45 is the amino acid sequence of murine TLR7 protein (P58681).

SEQ ID NO:46 is the nucleotide sequence of human TLR8 cDNA (AF245703).

SEQ ID NO:47 is the nucleotide sequence of human TLR8 cDNA (AF246971).

SEQ ID NO:48 is the nucleotide sequence of human TLR8 cDNA (NM_138636).

SEQ ID NO:49 is the nucleotide sequence of human TLR8 cDNA (NM_016610).

SEQ ID NO:50 is the amino acid sequence of human TLR8 protein (AAF78036).

SEQ ID NO:51 is the amino acid sequence of human TLR8 protein (AAF64061).

SEQ ID NO:52 is the amino acid sequence of human TLR8 protein (Q9NR97).

SEQ ID NO:53 is the amino acid sequence of human TLR8 protein (NP_619542).

SEQ ID NO:54 is the amino acid sequence of human TLR8 protein (NP_057694).

SEQ ID NO:55 is the nucleotide sequence of murine TLR8 cDNA (AY035890).

SEQ ID NO:56 is the nucleotide sequence of murine TLR8 cDNA (NM_133212).

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SEQ ID NO:57 is the amino acid sequence of murine TLR8 protein (AAK62677).

SEQ ID NO:58 is the amino acid sequence of murine TLR8 protein (NP_573475).

SEQ ID NO:59 is the amino acid sequence of murine TLR8 protein (P58682).

SEQ ID NO:60 is the nucleotide sequence of human TLR9 cDNA (AF245704).

5   SEQ ID NO:61 is the nucleotide sequence of human TLR9 cDNA (AB045180).

SEQ ID NO:62 is the amino acid sequence of human TLR9 protein (AAF78037).

SEQ ID NO:63 is the amino acid sequence of human TLR9 protein (AAF72189).

SEQ ID NO:64 is the amino acid sequence of human TLR9 protein (AAG01734).

SEQ ID NO:65 is the amino acid sequence of human TLR9 protein (AAG01735).

10   SEQ ID NO:66 is the amino acid sequence of human TLR9 protein (AAG01736).

SEQ ID NO:67 is the amino acid sequence of human TLR9 protein (BAB19259).

SEQ ID NO:68 is the nucleotide sequence of murine TLR9 cDNA (AF348140).

SEQ ID NO:69 is the nucleotide sequence of murine TLR9 cDNA (AB045181).

SEQ ID NO:70 is the nucleotide sequence of murine TLR9 cDNA (AF314224).

15   SEQ ID NO:71 is the nucleotide sequence of murine TLR9 cDNA (NM_031178).

SEQ ID NO:72 is the amino acid sequence of murine TLR9 protein (AAK29625).

SEQ ID NO:73 is the amino acid sequence of murine TLR9 protein (AAK28488).

SEQ ID NO:74 is the amino acid sequence of murine TLR9 protein (BAB19260).

SEQ ID NO:75 is the amino acid sequence of murine TLR9 protein (NP_112455).

20   SEQ ID NO:76 is the nucleotide sequence of human TLR10 cDNA (AF296673).

SEQ ID NO:77 is the amino acid sequence of human TLR10 protein (AAK26744).

SEQ ID NO:78 is the nucleotide sequence of human TLR6 cDNA (AB020807).

SEQ ID NO:79 is the nucleotide sequence of human TLR6 mRNA (NM_006068).

SEQ ID NO:80 is the amino acid sequence of human TLR6 protein (BAA78631).

25   SEQ ID NO:81 is the amino acid sequence of human TLR6 protein (NP_006059).

SEQ ID NO:82 is the amino acid sequence of human TLR6 protein (Q9Y2C9).

SEQ ID NO:83 is the nucleotide sequence of murine TLR6 cDNA (AB020808).

SEQ ID NO:84 is the nucleotide sequence of murine TLR6 cDNA (NM_011604).

SEQ ID NO:85 is the nucleotide sequence of murine TLR6 cDNA (AF314636).

30   SEQ ID NO:86 is the amino acid sequence of murine TLR6 protein (BAA78632).

SEQ ID NO:87 is the amino acid sequence of murine TLR6 protein (AAG38563).

SEQ ID NO:88 is the amino acid sequence of murine TLR6 protein (NP_035734).

SEQ ID NO:89 is the amino acid sequence of murine TLR6 protein (Q9EPW9).

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SEQ ID NO:90 is the nucleotide sequence of a consensus sequence for NF-κB p50 subunit.

SEQ ID NO:91 is the nucleotide sequence of a consensus sequence for NF-κB p65 subunit.

5   SEQ ID NO:92 is the nucleotide sequence of an example of an NF-κB p65 subunit binding site.

SEQ ID NO:93 is the nucleotide sequence of an example of a murine CREB binding site.

SEQ ID NO:94 is the nucleotide sequence of an example of a murine AP-1 binding

10   site.

SEQ ID NO:95 is the nucleotide sequence of an example of a murine AP-1 binding site.

SEQ ID NO:96 is the nucleotide sequence of an example of an ISRE.

SEQ ID NO:97 is the nucleotide sequence of an example of an ISRE.

15   SEQ ID NO:98 is the nucleotide sequence of an example of an ISRE.

SEQ ID NO:99 is the nucleotide sequence of an example of an ISRE.

SEQ ID NO:100 is the nucleotide sequence of an example of an ISRE.

SEQ ID NO:101 is the nucleotide sequence of an example of an ISRE.

SEQ ID NO:102 is the nucleotide sequence of an example of an ISRE.

20   SEQ ID NO:103 is the nucleotide sequence of an example of an SRE.

SEQ ID NO:104 is the nucleotide sequence of an example of an SRE.

SEQ ID NO:105 is the nucleotide sequence of an example of an SRE.

SEQ ID NO:106 is the nucleotide sequence of an example of an NFAT binding site.

SEQ ID NO:107 is the nucleotide sequence of an example of an NFAT binding site.

25   SEQ ID NO:108 is the nucleotide sequence of an example of an NFAT binding site.

SEQ ID NO:109 is the nucleotide sequence of an example of an NFAT binding site.

SEQ ID NO:110 is the nucleotide sequence of an example of a GAS.

SEQ ID NO:111 is the nucleotide sequence of a p53 binding site consensus sequence.

SEQ ID NO:112 is the nucleotide sequence of an example of a p53 binding site.

30   SEQ ID NO:113 is the nucleotide sequence of an example of a p53 binding site.

SEQ ID NO:114 is the nucleotide sequence of an example of a p53 binding site.

SEQ ID NO:115 is the nucleotide sequence of an example of a p53 binding site.

SEQ ID NO:116 is the nucleotide sequence of an example of a p53 binding site.

SEQ ID NO:117 is the nucleotide sequence of an example of a p53 binding site.

SEQ ID NO:118 is the nucleotide sequence of an example of a TARE (TNF-α response element).

SEQ ID NO:119 is the nucleotide sequence of an example of an SRF binding site.

SEQ ID NO:120 is the nucleotide sequence of an example of an SRF binding site.

SEQ ID NO:121 is the nucleotide sequence of the -620 to +50 promoter region of IFN-α4.

SEQ ID NO:122 is the nucleotide sequence of the -140 to +9 promoter region of IFN-α1.

SEQ ID NO:123 is the nucleotide sequence of the -140 to +9 promoter region of IFN-α1 (point mutation, AL353732).

SEQ ID NO:124 is the nucleotide sequence of the -280 to +20 promoter region of IFN-β.

SEQ ID NO:125 is the nucleotide sequence of the -397 to +5 promoter region of human RANTES (AB023652).

SEQ ID NO:126 is the nucleotide sequence of the -751 to +30 promoter region of human IL-12 p40.

SEQ ID NO:127 is the nucleotide sequence of the -250 to +30 promoter region of human IL-12 p40.

SEQ ID NO:128 is the nucleotide sequence of the -288 to +7 promoter region of human IL-6.

SEQ ID NO:129 is the nucleotide sequence of the IL-6 gene promoter from -1174 to +7 (M22111).

SEQ ID NO:130 is the nucleotide sequence of the -734 to +44 promoter region derived from human IL-8.

SEQ ID NO:131 is the nucleotide sequence of the -162 to 44 promoter region of human IL-8.

SEQ ID NO:132 is the nucleotide sequence of the -615 to +30 promoter region of human TNF-α.

SEQ ID NO:133 is the nucleotide sequence of a promoter region of human TNF-β.

SEQ ID NO:134 is the nucleotide sequence of the -875 to +97 promoter region of human IP-10.

SEQ ID NO:135 is the nucleotide sequence of the -219 to +114 promoter region of human CXCL11 (IP-9).

SEQ ID NO:136 is the nucleotide sequence of the full length promoter region of human CXCL11 (IP-9).

SEQ ID NO:137 is the nucleotide sequence of the -289 to +217 promoter region of IGFBP4 (Insulin growth factor binding protein 4).

SEQ ID NO:138 is the nucleotide sequence of the full length promoter region of IGFBP4.

SEQ ID NO:139 is the nucleotide sequence of an immunostimulatory nucleic acid.

SEQ ID NO:140 is the nucleotide sequence of an immunostimulatory nucleic acid.

SEQ ID NO:141 is the nucleotide sequence of an immunostimulatory nucleic acid.

SEQ ID NO:142 is the nucleotide sequence of an immunostimulatory nucleic acid.

SEQ ID NO:143 is the nucleotide sequence of an immunostimulatory nucleic acid.

SEQ ID NO:144 is the nucleotide sequence of an immunostimulatory nucleic acid.

SEQ ID NO:145 is the nucleotide sequence of an immunostimulatory nucleic acid.

SEQ ID NO:146 is the nucleotide sequence of an immunostimulatory nucleic acid.

SEQ ID NO:147 is the nucleotide sequence of an immunostimulatory methylated CpG nucleic acid.

SEQ ID NO:148 is the nucleotide sequence of an immunostimulatory methylated CpG nucleic acid.

SEQ ID NO:149 is the nucleotide sequence of an immunostimulatory methylated CpG nucleic acid.

SEQ ID NO:150 is the nucleotide sequence of an immunostimulatory methylated CpG nucleic acid.

SEQ ID NO:151 is the nucleotide sequence of an immunoinhibitory nucleic acid.

SEQ ID NO:152 is the nucleotide sequence of a sense primer for human TLR3.

SEQ ID NO:153 is the nucleotide sequence of an antisense primer for human TLR3.

SEQ ID NO:154 is the nucleotide sequence of a GpC nucleic acid.

SEQ ID NO:155 is the nucleotide sequence of a CpG ODN.

SEQ ID NO:156 is the nucleotide sequence of a GpC ODN.

SEQ ID NO:157 is the nucleotide sequence of a Me-CpG ODN.

SEQ ID NO:158 is the nucleotide sequence of a TLR9 ligand.

SEQ ID NO:159 is the nucleotide sequence of a TLR9 ligand.

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SEQ ID NO:160 is the nucleotide sequence of a TLR9 ligand.

SEQ ID NO:161 is the nucleotide sequence of a TLR9 ligand.

Detailed Description of the Invention

In its broadest sense, the invention relates to screening methods and tools to be used to identify and discriminate between newly discovered immunomodulatory molecules and to compare and standardize compositions of known immunomodulatory molecules. The immunomodulatory molecules are preferably TLR ligands.

Thus, the invention is based in part on the discovery that cell lines expressing endogenous TLR respond to TLR ligands in a manner similar to the response of peripheral blood mononuclear cells (PBMC). PBMC respond to immunomodulatory TLR ligands by modulating one or more parameters including gene expression, cell surface marker expression, cytokine and/or chemokine production and secretion, cell cycle status, phosphorylation status, and the like. TLR ligands can be categorized and distinguished based on the cellular changes they induce (i.e., their induction profiles). The ability of a TLR ligand to provide therapeutic or prophylactic benefit to a subject depends on its induction profile. The ability to screen new TLR ligands for a panel of response indicators or parameters allows for rapid discrimination and categorization of TLR ligands. Moreover, the similarity between the cell line responses and those observed after in vivo administration of the TLR ligand indicates that the cell lines are suitable predictors of in vivo activity. The use of in vitro propagated cell lines additionally overcomes the variability encountered when using freshly isolated PBMC.

The TLR ligands identified according to the invention therefore can be used therapeutically or prophylactically in a more patient- or disorder-specific manner. The invention allows for the tailoring of TLR ligands for particular patients or disorders.

The invention identifies a number of cell lines that can be used to identify TLR ligands based on endogenous TLR expression such as TLR3, TLR7 and TLR9 expression. As an example, the invention is premised in part on the discovery of TLR9 expression in a number of cell lines including RPMI 8226, Raji, RAMOS, THP-1, Nalm-6 and KG-1. Cell lines RPMI 8226, Raji and RAMOS have been determined to express TLR7 according to the invention. Cell lines KG-1 cell, a Nalm cell, a Raji cell, a RAMOS cell, a Jurkat cell, a Hela cell, a Hep-2 cell, a Hep-2 cells, a A549 cell, a Bewo cell, an NK-92 cell or an NK-92 MI cell have been discovered to express TLR3 according to the invention.

It is further premised in part on the discovery that RPMI 8226 cells respond to the imidazoquinoline compound R-848. Consistent with this latter finding, it was also discovered that RPMI 8226 cells express TLR7.

The invention in other aspects provides for screening methods and tools for verifying and standardizing compositions containing known TLR ligands. These compositions may be for example commercial production lots to be used in a clinical setting. Accordingly, the invention provides methods for standardizing lots of known TLR ligands prior to distribution and use clinically. In this way, production processes can be observed and controlled and substandard production lots can be identified and eliminated prior to shipment.

The methods of the instant invention can be used at any step in the preparation and production of clinical material, i.e., pharmaceutical product. In particular, the methods will find use in characterizing or validating raw materials, in-process materials, finished product materials (e.g., pre-release materials), and post-production materials (e.g., post-release materials). The methods can also be used to validate existing process methods, as well as to validate new or changed process methods used in the production of the pharmaceutical product.

Screening Assays Generally

The screening assays provided herein may be used to identify immunomodulatory agents. Immunomodulatory agents are agents that either stimulate or inhibit immune responses in a subject. Accordingly, as used herein, immunomodulation embraces both immunostimulation and immunoinhibition.

The screening methods are used to identify TLR agonists and antagonists. The methods can also be used to identify compounds that enhance the immunostimulation induced by a TLR agonist. This latter set of compounds is referred to herein as "enhancers". A TLR agonist is a compound that stimulates TLR signaling activity. A TLR antagonist is a compound that inhibits TLR signaling activity. Agonists are generally referred to herein as immunostimulatory compounds because stimulation of TLR is associated with immune stimulation. Antagonists are generally referred to herein as immunoinhibitory compounds because inhibition of TLR is associated with immune inhibition. TLR antagonists include compounds that reduce (or eliminate completely) the immunostimulation induced by a TLR

agonist. In some embodiments, the agonists, antagonists and enhancers are TLR ligands (i.e., they bind to a TLR). In other embodiments, the test compounds with agonist, antagonist or enhancer activity may act downstream or upstream of the TLR-TLR ligand interaction.

An "immunostimulatory compound" as used herein refers to a natural or synthetic
5   compound that characteristically induces a TLR-mediated response when contacted with a suitable functional TLR polypeptide. In one embodiment the immunostimulatory compound is a natural or synthetic compound that induces a TLR-mediated response when contacted with a cell that naturally or artificially expresses a suitable functional TLR polypeptide. Depending on the aspect of the invention, the cell may be an experimental cell or a primary
10   cell such as a PBMC.

Examples of immunostimulatory compounds include the following immunostimulatory nucleic acids, which are discussed in further detail below:

5'-TCGTCGTTTTGTCGTTTTGTCGTT-3'	(SEQ ID NO:1)
5'-TCGTCGTTTTGACGTTTTGTCGTT-3'	(SEQ ID NO:139)
5'-TCGTCGTTTTGTCGTTTTTTTCGA-3'	(SEQ ID NO:140)
5'-TCGTCGTTTCGTCGTTTCGTCGTT-3'	(SEQ ID NO:141)
5'-TCGTCGTTTCGTCGTTTTGTCGTT-3'	(SEQ ID NO:142)
5'-TCGTCGTTTTTCGGTCGTTTT-3'	(SEQ ID NO:143)
5'-TCGTCGTTTTTCGTGCGTTTTT-3'	(SEQ ID NO:144)
5'-TCGTCGTTTTCGGCGGCCGCCG-3'	(SEQ ID NO:145)
5'-TCGTC_GTTTTAC_GGCGCC_GTGCCG-3'	(SEQ ID NO:146)

Imidazoquinolines are immune response modifiers thought to induce expression of several cytokines including interferons (e.g., IFN-α and IFN-β), TNF-α and some interleukins
25   (e.g., IL-1, IL-6 and IL-12) as well as chemokines (e.g., IP-10 and IL-8). Imidazoquinolines are capable of stimulating a Th1 immune response, as evidenced in part by their ability to induce increases in IgG2a levels. Imidazoquinoline agents reportedly are also capable of inhibiting production of Th2 cytokines such as IL-4, IL-5, and IL-13. Some of the cytokines induced by imidazoquinolines are produced by macrophages and dendritic cells. Some
30   species of imidazoquinolines have been reported to increase NK cell lytic activity and to stimulate B cells proliferation and differentiation, thereby inducing antibody production and secretion. Imidazoquinoline mimics can also be tested using the screening methods.

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An "immunoinhibitory compound" as used herein refers to a natural or synthetic compound that characteristically inhibits a TLR-mediated response when contacted with a suitable functional TLR polypeptide. In one embodiment the immunoinhibitory compound is a natural or synthetic compound that inhibits a TLR-mediated response when contacted with a

5   cell that naturally or artificially expresses a suitable functional TLR polypeptide.

In addition to the immunoinhibitory nucleic acids disclosed elsewhere herein, immunoinhibitory compounds and TLR antagonists encompass certain small molecules (chloroquine, quinacrine, 9-aminoacridines and 4-aminoquinolines, and derivatives thereof) described by Macfarlane and colleagues in U.S. Pat. 6,221,882; U.S. Pat. 6,399,630; U.S. Pat.

10   6,479,504; U.S. Pat. 6,521,637; and published U.S. Pat. application 2002/0151564, the contents of all of which are hereby incorporated by reference in their entirety.

The invention provides in part methods and tools that utilize cell lines, in modified or unmodified form, as surrogates for PBMC. Immunomodulation by TLR ligands can be assessed using one or preferably more parameters including but not limited to cytokine and

15   chemokine secretion, upregulation of cell surface markers, changes in cell proliferation, phosphorylation changes, and the like. These parameters may be native readouts or artificial readouts as described herein.

The cellular response to immunostimulatory nucleic acids by the cell lines described herein (e.g., RPMI 8226, Raji, RAMOS, and the like) so resembles that of PBMC that these

20   cells can be used to identify and differentiate between immunomodulatory compounds based on the extent of the induced response and the particular profile of that response. The invention provides a number of cell lines each with a particular endogenous TLR expression profile, as described herein.

The cell lines can be used to identify immunomodulatory compounds with particular

25   response profiles. As an example, the cell lines can be used to identify molecules that are mimics to known TLR ligands. The cell lines can also be used to identify TLR ligands that trigger some but not necessarily all of the responses induced by known TLR ligands. For example, the cell line can be used to distinguish between compounds based on individual or group cytokine or chemokine secretion, or based on upregulation of one, a subset or all cell

30   surface markers. As an example, in some therapeutic instances, it may be desirable to use a compound that induces the secretion of relatively high levels of chemokine such as IP-10, yet induces only relatively low levels of one or more other factors. The screening methods of the invention allow for the identification of such a compound with this type of induction profile.

It is to be understood that the screening method also can be used to determine effective amounts of known and newly identified immunomodulatory compounds. For example, the EC_{50} value of a TLR ligand for the production of a particular cytokine or chemokine can be determined, thereby facilitating comparison between different nucleic acids.

5   Generally, these assays require the incubation of cells with a reference compound and a test compound, and an analysis of the readout. Depending on the embodiment, the same cells are exposed to the reference compound and the test compound. An example of this latter embodiment is a screening assay for compounds that enhance the immunostimulatory effects of a TLR agonist. Another example is a screening assay for compounds that inhibit the

10   immunostimulatory effects of a TLR agonist. In both examples, the reference compound is a positive reference compound (i.e., it is itself immunostimulatory).

In other embodiments, particularly those directed at identifying immunostimulatory compounds, separate aliquots from the same cell line (or from the same freshly harvested cell population) are exposed to either the reference compound or the test compound, and the

15   readouts from each are measured and compared to the other. If the reference compound is a negative reference compound (i.e., it is inert and neither immunostimulatory nor immunoinhibitory), then any test level that is greater than the reference level is indicative of a test compound that has at least some immunostimulatory capacity. Generally, the negative reference compound is used to set background levels of immunostimulation or

20   immunoinhibition observed in the absence of the test compound. If the reference compound is a positive reference compound (i.e., it is immunostimulatory), then it is possible to compare and contrast the induction profile of the test compound to that of the reference compound.

In some instances, separate reference assays individually containing a positive and a negative reference compound are performed alongside the test assay. For example, if the test

25   assay is a screen for an immunostimulatory TLR ligand, then reference assays can be a positive reference assay (in which the reference compound is immunostimulatory), a negative reference assay (in which the reference compounds is immunologically inert or neutral), or both. A test compound is defined as immunostimulatory if it induces a response greater than that of the negative reference compound. The level and profile of the immunostimulatory

30   response can be compared to the level and profile induced by the positive reference compound. It is to be understood that a test compound that induces a level of immunostimulation less than that of the positive reference compound may still be considered immunostimulatory according to the invention. Modifications to these screening assays for a

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desired readout will be apparent to those of ordinary skill in the are based on the teachings provided herein.

If the test assay is a screen for an immunoinhibitory TLR ligand, then the assay may generally involve co-incubation of the test compound and a positive reference compound.

5   The control assay may include co-incubation of the negative and positive reference compounds. As used herein, co-incubation embraces simultaneous or consecutive addition of the reference and test compounds. The test compound may be added before or after the positive reference compound. An immunoinhibitory test compound may be identified by a diminution of the immunostimulatory response induced by the positive reference compound

10   when in the presence of the test compound. If the level of the response is less in the presence of the test compound, this indicates that the test compound is capable of interfering with the immunostimulatory effects of the positive reference compound. As an example, simultaneous or consecutive addition of a putative immunoinhibitory test compound can reduce the amount of cytokines or chemokines secreted by cells in response to the positive reference compound

15   alone, indicating an inhibition of the immunostimulatory effects of the positive reference compound.

The reference immunoinhibitory compound can be used at one or more concentrations in conjunction with a selected or constant concentration of reference immunostimulatory compound. Under proper conditions, the immunostimulatory effect of the reference

20   immunostimulatory compound will be less in the presence of the immunoinhibitory substance than in the absence of the immunoinhibitory substance. Furthermore, under proper conditions, the immunostimulatory effect of the reference immunostimulatory compound will decrease with increasing concentration of the immunoinhibitory substance.

The breadth of response by the cell line to immunomodulatory compounds, and its

25   facile manipulation, allows for the identification of novel compounds. The cell line allows the rapid discovery of such compounds given that is lends itself to high throughput screening methods such as those provided herein. These methods and compositions are described in greater detail below.   The invention therefore provides screening methods that utilize cell lines that either endogenous express TLRs such as the RPMI 8226 cell line as well as cell

30   lines that have been modified to express TLRs. The invention further provides compositions that comprise such cell lines.

The verification and standardization methods of the invention generally involve assays in which an isolated cell expressing a functional TLR is contacted with each of two

compositions, each composition containing a known ligand for the TLR. One composition is a reference composition, and the assay using the reference composition yields a reference activity. The second composition is a test composition, and the assay using the test composition yields a test activity. The two contacting steps can be performed on separate

5　cells that are alike, and typically will be performed on separate populations of cells that are alike. For example, the separate cells or the separate populations of cells can be drawn from a single population of cells. In typical usage according to this embodiment, the reference and test activities are measured essentially concurrently, although the use of historical reference activity is also contemplated by the methods of the invention. As an alternative, the two

10　contacting steps can be performed on a single cell or on a single population of cells, usually in an essentially concurrent manner when it is desirable to have competition between reference and test compositions. In one embodiment the known TLR ligand is a nucleic acid molecule.

　　　　The assays of the invention are performed under specific conditions so that comparison can be made between reference and test activities or levels. The results of the

15　comparison can be used as a basis upon which to accept or reject the test material as suitable for its intended use.

　　　　The biological characterization of the reference composition will generally entail a series of biological activity measurements of the reference composition using a single assay under defined conditions in order to define a range of inter-test variance. The range of inter-

20　test variance so obtained using reference composition can be used to define an acceptable range of variance within which a subsequent test measurement must fall in order to satisfy quality standards. Such a range of acceptable variance can serve as a basis for developing predetermined range of variance about the reference activity, i.e., acceptance criteria for a particular test composition or test lot. For example, a particular reference composition can be

25　assayed under defined conditions in a number of independent measurements and found to yield a result expressed as 100 ± 5 units of activity. Under this same example, a subsequent test measurement of a test composition performed using the same assay and defined conditions is found to yield 97 units of activity. The activity of the test composition under this example thus yielded a result that falls within the normal range of inter-test variance

30　observed for the reference composition. Accordingly, the test material under this example could be selected on the basis of the test activity falling within a predetermined range of variance about the reference activity. In short, the test material can be deemed acceptable

provided the test activity falls within a predetermined range of activity that is related to the activity of the reference material.

In one embodiment, the methods of the invention provide for comparison between a reference lot of a particular TLR ligand and a test lot of the same particular TLR ligand. Such
5   comparison is useful for quality control assessment of the test lot of material, also referred to herein as validation, e.g., product validation. Such comparison is also useful for process validation.

In another embodiment, the methods of the invention provide for comparison between a reference lot of a particular TLR ligand and a test lot of a different TLR ligand. In a simple
10   example, where a test TLR ligand (T) is expected to have little or no activity characteristic of reference TLR ligand (R), comparison can be made between T and R to confirm the lack of R-like activity possessed by T. In a more complex example, where a test TLR ligand (C) is capable of exerting two different effects, wherein each effect is characteristic of one of two different classes of TLR ligand and is best characterized by one of two different reference
15   TLR ligands (A and B), the test TLR ligand (C) can be compared with either of the two reference TLR ligands (A or B). In this second example, test composition C could be found, for example, to possess 50 percent A-like activity compared with reference A and 70 percent B-like activity compared with reference B. Test composition C could thus independently meet or fail to meet predetermined standards for each of A-like activity and B-like activity.
20   Such comparison is also useful for quality control assessment of the test lot of material, e.g., product validation. Of course test TLR ligand C can alternatively or additionally be compared against reference TLR ligand C, as described in the preceding paragraph.

To facilitate the methods of the invention, certain conditions for carrying out the assays are standardized and used for measurements of both reference activity and test activity.
25   In this way direct comparison between reference activity and test activity can be made readily. Conditions that can be standardized and used in this manner can include, without limitation, readout, temperature, media characteristics, duration (time between introduction of reference composition or test composition and activity measurement), methods of sampling, etc. In some embodiments the methods of the invention can be at least partially automated in order to
30   increase throughput and/or to reduce inter-test variability. For example, robotic devices and workstations with the capacity to dispense and/or sample fluids in a set or programmable fashion are now well known in the art and can be used in performing the methods of the instant invention.

In one embodiment a standard curve of reference composition activity is employed. Typically the standard curve is generated by selecting conditions including concentration of the reference composition such that the dose-response curve is essentially linear (and the slope is non-zero) over a range of concentrations that includes the effective concentration at

5 which activity is 50 percent of maximum (EC50). In one embodiment the standard curve spans a range of concentrations defined by EC50 $\pm$ 1 log concentration, e.g., 1×10^{-7} M – 1×10^{-5} M, where EC50 is 1×10^{-6} M. In another embodiment the standard curve spans a broader range of concentrations defined by EC50 $\pm$ 2 log concentration, e.g., 1×10^{-8} M – 1×10^{-4} M, where EC50 is 1×10^{-6} M. In yet another embodiment the standard curve spans a narrower

10 range of concentrations defined by EC50 $\pm$ 0.5 log concentration, e.g., 3.16×10^{-7} M – 3.16×10^{-6} M, where EC50 is 1×10^{-6} M. The foregoing embodiments are intended to be exemplary and not limiting in any way. One of skill in the art will be able to select, for a given reference composition and without undue experimentation, an appropriate range of concentrations about some middle value in order to generate an essentially linear standard curve with a non-zero

15 slope.

In one embodiment a non-linear standard curve of reference and test composition activity is employed. The standard curve can be generated by selecting conditions including concentrations of the reference composition such that the dose-response curve is sigmoidal and the EC50 value can be determined. Comparison of reference and test activity can be done

20 by comparing, e.g., the EC50 values of both curves. Concentration range is chosen to yield a complete sigmoidal response, e.g., concentration should include EC50 $\pm$ 3 log concentration or EC50 $\pm$ 4 log concentration. In the case of testing an inhibitory compound the value determined would be the IC50, i.e., concentration where inhibition of the stimulatory signal is half-maximal.

25 The methods of the invention can be adapted to be automated or at least partially automated methods, as well as to parallel array or high throughput format methods. For example, the assays can be set up using multiwell plates in which cells are dispensed in individual wells and reagents are added in a systematic manner using a multiwell delivery device suited to the geometry of the multiwell plate. Manual and robotic multiwell delivery

30 devices suitable for use in a high throughput screening assay are known by those skilled in the art. Each well or array element can be mapped in a one-to-one manner to a particular test condition, such as the test compound. Readouts can also be performed in this multiwell array, preferably using a multiwell plate reader device or the like. Examples of such devices are

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known in the art and are available through commercial sources. Sample and·reagent handling
can be automated to further enhance the throughput capacity of the screening assay, such that
dozens, hundreds, thousands, or even millions of parallel assays can be performed in a day or
in a week. Fully robotic systems are known in the art for applications such as generation and
5   analysis of combinatorial libraries of synthetic compounds. See, for example, U.S. Pat. Nos.
5,443,791 and 5,708,158.

Cell lines

The screening methods may use experimental cells. As used herein, an experimental
10   cell is a non-primary cell (i.e., it is not a cell that has been recently harvested from a subject).
It excludes, for example, freshly harvested PBMCs. An experimental cell includes a cell from
a cell line such as the RPMI 8226 cell line.

In certain embodiments, the cell naturally expresses a functional TLR. In one
embodiment relating to the verification and standardization aspects of the invention, the cell
15   may be a PBMC, preferably a PBMC freshly harvested from a subject.

Cells that would be suitable for identification of TLR agonists, antagonists or
enhancers according to the invention may possess one or more particular attributes. These
attributes include but are not limited to being of human origin, being an immortalized stable
cell line, endogenously expressing at least one functional TLR or a combination of functional
20   TLRs, having intact signaling mechanisms, having intact uptake mechanisms, being able to
upregulate cytokines, chemokines or cell surface markers, deriving from normal human B
cells or from myeloma or B cell leukemia, deriving from human plasmacytoid and myeloid
dendritic cells, and readily activatable by TLR ligands such as TLR7 ligands, TLR8 ligands or
TLR9 ligands such as CpG nucleic acids or nucleic acids having other immunostimulatory
25   sequence motifs or small molecules such as imidazoquinoline compounds.

In some embodiments, the cell line is the Raji cell line which expresses TLR3, TLR7
and TLR9. This latter cell line secretes, for example, IL-6 and IFN-α2 upon CpG nucleic acid
exposure. In other embodiments, the cell line is RPMI 8226 which expresses TLR7 and
TLR9. Upon CpG nucleic acid exposure, this cell line expresses, produces and/or secretes IL-
30   8, IL-10, IP-10 and TNF-α. It also expresses at its cell surface CD86, HLA-DR and CD71.
In yet other embodiments, the cell line is the RAMOS cell line which expresses TLR3, TLR7
and TLR9. This cell line at least induces CD80 cell surface expression in response to CpG
nucleic acid exposure.

The cell lines have been observed to respond in a concentration dependent manner to TLR ligands such as but not limited to CpG nucleic acids and some non-CpG nucleic acids including T-rich nucleic acids, poly-T nucleic acids and poly-G nucleic acids. The highest responses have been observed using CpG nucleic acids.

5   The screening methods employ a variety of cell lines as shown in the Examples. These include A549 (human lung carcinoma, ATCC CCL-185), BeWo (human choriocarcinoma, ATCC CCL-98), HeLa (human cervix carcinoma, ATCC CCL-2), Hep-2 (human cervix carcinoma, ATCC CCL-23), KG-1 (human acute myeloid leukemia, ATCC CCL-246), MUTZ-3 (human acute myelomonocytic leukemia, German Collection of Cell

10   lines and Microorganisms (DSZM) ACC-295), Nalm-6 (human B cell precursor leukemia, DSZM ACC-128), NK-92 (human Natural killer cell line, ATCC CRL-2407), NK-92 MI (IL-2 independent human Natural killer cell line, ATCC CRL-2408), Raji (human B lymphocyte Burkitt's lymphoma, ATCC CCL-86), RAMOS (B lymphocyte Burkitt's lymphoma, ATCC CRL-1596), RPMI 8226 (human B lymphocyte multiple myeloma, ATCC CCL-155), THP-1

15   (human acute monocytic leukemia, ATCC TIB 202), U937 (human lymphoma, ATCC CRL-1593.2) and Jurkat (human T cell leukemia, ATCC TIB 152).

As shown in the Examples, each of the afore-mentioned cell lines has a particular endogenous TLR expression profile which dictates its suitability in a particular screening assay.

20   A cell that artificially expresses a functional TLR can be a cell that does not express the functional TLR but for a transfected TLR expression vector. For example, human 293 fibroblasts (ATCC CRL-1573) do not express TLR7, TLR8 or TLR9, and they express very little TLR3. As described in the examples below, such cells can be transiently or stably transfected with suitable expression vector (or vectors) so as to yield cells that do express

25   TLR3, TLR7, TLR8, TLR9, or any combination thereof. Alternatively, a cell that artificially expresses a functional TLR can be a cell that expresses the functional TLR at a significantly higher level with the TLR expression vector than it does without the TLR expression vector. Transfected cells are considered modified cells, as used herein.

A cell that artificially expresses an expression or reporter construct is preferably stably

30   transfected.

RPMI

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The RPMI 8226 cell line is a human multiple myeloma cell line. The cell line was established from the peripheral blood of a 61 year old man at the time of diagnosis for multiple myeloma (IgG lambda type). RPMI 8226 was previously reported as responsive to CpG nucleic acids as evidenced by the production and secretion of IL-6 protein and production of IL-12p40 mRNA. (Takeshita et al. (2000), Eur. J. Immunol. 30, 108-116, and Takeshita et al. (2000) *Ibid.* 30, 1967-1976) Takeshita et al. however used the cell line solely to study promoter constructs in order to identify transcription factor binding sites important for CpG nucleic acid signaling. It is now known according to the invention that the cell line produces a number of other chemokines and cytokines including IL-8, IL-10 and IP-10. It has also been discovered according to the invention that the cell line responds to immunostimulatory nucleic acids by upregulating cell surface expression of particular markers. Many of these markers, including CD71, CD86 and HLA-DR, are similarly upregulated in PBMCs exposed to immunostimulatory nucleic acids. This has been observed using flow cytometric analysis of the cell line following CpG nucleic acid exposure. In other aspects of the invention, the cell line can be used in similar screening assays that involve secretion of IL-6, IL-12 and/or TNF-α.

It has recently been discovered that R-848 mediates its immunostimulatory effects via other TLR family members, namely TLR7 and TLR8. TLR7 has previously been found expressed on human B cells. It has now also been discovered according to the invention that RPMI 8226 expresses TLR9 as well as TLR7, thus making it a suitable cell line for identifying immunostimulatory nucleic acid and/or imidazoquinoline (e.g., R-848) mimics or other small molecules that also signal through TLR7 and/or TLR9. Incubation of RPMI 8226 cells with the imidazoquinoline R-848 (Resiquimod) induces for example IL-8, IL-10 and IP-10 production.

Known TLR Ligands

Ligands for many but not all of the TLRs have been described. For instance, it has been reported that TLR1 and TLR2 signals in response to peptidoglycan and lipopeptides. Yoshimura A et al. (1999) *J Immunol* 163:1-5; Brightbill HD et al. (1999) *Science* 285:732-6; Aliprantis AO et al. (1999) *Science* 285:736-9; Takeuchi O et al. (1999) *Immunity* 11:443-51; Underhill DM et al. (1999) *Nature* 401:811-5. TLR4 has been reported to signal in response to lipopolysaccharide (LPS). Hoshino K et al. (1999) *J Immunol* 162:3749-52; Poltorak A et al. (1998) *Science* 282:2085-8; Medzhitov R et al. (1997) *Nature* 388:394-7. Bacterial

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flagellin has been reported to be a natural ligand for TLR5. Hayashi F et al. (2001) *Nature* 410:1099-1103. TLR6, in conjunction with TLR2, has been reported to signal in response to proteoglycan. Ozinsky A et al. (2000) *Proc Natl Acad Sci USA* 97:13766-71; Takeuchi O et al. (2001) *Int Immunol* 13:933-40.

5　　　　　TLR9 is a receptor for CpG DNA. Hemmi H et al. (2000) *Nature* 408:740-5. Other TLR9 ligands are described herein under "Immunostimulatory Nucleic Acids". Certain imidazoquinoline compounds having antiviral activity are ligands of TLR7 and TLR8. Imidazoquinolines are potent synthetic activators of immune cells with antiviral and antitumor properties. R-848 is a ligand for human TLR7 and TLR8. Jurk M et al. (2002) *Nat Immunol*

10　　3:499. Ligands of TLR3 include poly(I:C) and double-stranded RNA (dsRNA). Alexopoulou et a. (2001) Nature 413:732-738. For purposes of this invention, poly(I:C) and double-stranded RNA (dsRNA) are classified as oligonucleotide molecules. TLR3 may have a role in host defense against viruses.

15　　Reference and Test Compounds

　　　　　A test and/or reference compound can be a nucleic acid such as an oligonucleotide or a polynucleotide, an oligopeptide, a polypeptide, a lipid such as a lipopolysaccharide, a carbohydrate such as an oligosaccharide or a polysaccharide, or a small molecule. Alternatively, these compounds may also comprise or be synthesized from elements such as

20　　amino acids, carbohydrates, hormones, lipids, organic molecules, and the like.

　　　　　Small molecules in general include naturally occurring, synthetic, and semisynthetic organic and organometallic compounds with molecular weight less than about 2.5 kDa. Examples of small molecules include most drugs, subunits of polymeric materials, and analogs and derivatives thereof.

25　　　　　Some specific examples of small molecules include the imidazoquinolines. As used herein, an imidazoquinolines include imidazoquinoline amines (imidazoquinolinamines), imidazopyridine amines, 6,7-fused cycloalkylimidazopyridine amines, and 1,2 bridged imidazoquinoline amines. These compounds have been described in U.S. Pat. Nos. 4,689,338; 4,929,624; 5,238,944; 5,266,575; 5,268,376; 5,346,905; 5,352,784; 5,389,640;

30　　5,395,937; 5,482,936; 5,494,916; 5,525,612; 6,039,969 and 6,110,929. Particular species of imidazoquinoline agents include resiquimod (R-848; S-28463; 4-amino-2 ethoxymethyl-α,α-dimethyl-1*H*-imidazo[4,5-c]quinoline-1-ethanol); and imiquimod (R-837; S-26308; 1-(2-methylpropyl)-1*H*-imidazo[4,5-c]quinoline-4-amine). Further examples of specific small

molecules include 4-aminoquinoline and derivatives thereof, 9-aminoacridine and derivatives thereof, and additional compounds disclosed in U.S. Pat. Nos. 6,221,882; 6,399,630; 6,479,504; and 6,521,637; and published U.S. Pat. Application No. 2002/0151564 A1, the entire contents of which are hereby incorporated by reference.

5   The test and reference compounds may be formulated for pharmaceutical use or not. For example, a test compound not formulated for pharmaceutical use can be a compound (e.g., a lot or batch of the compound) under evaluation for possible use in preparing a pharmaceutical formulation of the compound.

          A reference compound, as used herein, is a compound having a known activity in the
10   presence of a TLR. The reference compound may stimulate TLR signaling (and is therefore regarded as a positive reference compound), or it may be inert in the presence of a TLR (and is therefore regarded as a negative reference compound). If it is a positive reference compound, it need not be the best known stimulator of TLR signaling (i.e., it is possible that other reference compounds and even test compounds will stimulate TLR signaling to a greater
15   extent). The readout of the screening assay may simply be stated relative to the level of signaling that occurs in the presence of the reference compound. Preferably, the reference compound is analyzed prior to the screening assay in order to determine its level of activity on a TLR. In some aspects of the invention, the reference compound and the test compound will be assayed separately (i.e., in separate wells); in other aspects, the reference compound and
20   the test compound will be assayed together (i.e., in the same well). These latter aspects are designed to measure the ability of a test compound to modulate the activity of the reference compound. The activity of the test compound and the reference compound combined (i.e., when assayed together in the same well) may be the same as that of the positive reference compound alone, indicating at a minimum that the test compound is not inhibitory; or it may
25   be less than that of the positive reference compound, indicating at a minimum that it is inhibitory to the effect of the reference compound; or it may be additive or synergistic possibly indicating that the test compound is an enhancer. The effect of an enhance may be due to its ability to stimulate TLR signaling independently of the positive reference compound.

30   A "reference composition" as used herein refers to a composition that includes a reference compound and optionally another agent, e.g., a pharmaceutically acceptable carrier and/or another biologically active agent. A reference compound may be an immunostimulatory compound or it may be an immunoinhibitory compound.

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As discussed further below, in some aspects of the invention the reference compositions include both finished products, e.g., finished pharmaceutical products, as well as raw materials and other in-process materials used for the preparation of such finished products, all of which contain a known TLR ligand. As used herein, a "production lot" shall

5   refer to a batch or lot of a completed product prepared for release as clinical material, e.g., a pharmaceutical product. As used herein, an "in-process lot" shall refer to a batch or lot of unfinished product that is prepared in the course of making a production lot; an "in-process lot" shall also refer to a batch or lot of raw material provided for use in the production of a production lot.

10   In some aspects of the invention, the reference compositions of the invention are highly characterized in terms of their chemical, physical, and biological properties. A reference composition will be a specific composition previously determined to have a specific activity, or range of specific activity, of the particular known TLR ligand present in the composition. As used herein, "specific activity" refers to an amount of activity per unit mass

15   or per unit volume of the reference composition as a whole, as determined using a defined assay under defined conditions. In one embodiment the reference composition is a representative sample of a particular lot or batch of a specific TLR ligand. In one embodiment the reference composition is a representative sample of a particular lot or batch of a specific TLR ligand formulated for pharmaceutical use, e.g., a sterile solution of the TLR

20   ligand at a determined concentration or activity.

At least the following parameters are typically very well defined for a given reference composition: chemical formula of the active ingredient TLR ligand (e.g., nucleobase sequence and type of backbone of a nucleic acid; structural formula of a small molecule); concentration; diluent composition; and purity. Such parameters as purity and concentration

25   can be determined using any appropriate physicochemical method, e.g., optical spectroscopy including absorbance at one or more specified wavelengths; nuclear magnetic resonance (NMR) spectroscopy; mass spectrometry (MS), including matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS); melting point; specific gravity; chromatography including as appropriate high pressure liquid chromatography (HPLC), one-

30   and two-dimensional polyacrylamide gel electrophoresis (PAGE), capillary electrophoresis, and the like; as well as other methods known to those of skill in the art.

Reference compositions can also be very well characterized in terms of their biological activity, independent of the methods of the invention, although the methods of the

invention generally include such characterization, at least in part. A reference composition can be very well characterized in terms of its biological activity by characterizing, both qualitatively and quantitatively, the response by sensitive cells to the reference composition under defined conditions. For example, a reference composition can be a specific CpG

5   oligonucleotide such as SEQ ID NO:1 which in a specific assay and under specific conditions of temperature, concentration, duration of contact between the CpG oligonucleotide and a population of TLR9-expressing cells, and particular readout, reliably yields a specific result or range of results. Results can be expressed in any suitable manner, but can include results expressed on a per-cell basis, e.g., picograms of particular cytokine per cell per hour of

10   contact with the reference composition. Reference compositions can be very well characterized in terms of their biological activity according to one or more parameters, for example, according to their capacity to induce each of a plurality of cytokines.

The methods of the invention also involve measurement of a test activity of a test composition containing a known TLR ligand. A "test composition" as used herein refers to a

15   composition that includes a test compound and optionally another agent, e.g., a pharmaceutically acceptable carrier and/or another biologically active agent. A test compound can be an immunostimulatory compound or it can be an immunoinhibitory compound. In some aspects of the invention, the test compound is a known TLR ligand. Test compositions of the invention may comprise known TLR agonist or TLR antagonist

20   compounds, generally but not necessarily nominally the same as the reference compositions against which comparison is to be made according to some aspects of the invention. Thus test compositions may encompass immunostimulatory compounds, immunoinhibitory compounds, known TLR ligands, finished pharmaceutical products, and raw materials and other in-process materials used for the preparation of such finished products.

25   Unlike a reference composition, a test composition is not characterized at all, or is only partially characterized, or is not as well characterized as the reference composition, in terms of its chemical, physical, or (most particularly) biological properties. The methods of the invention permit further characterization of the test composition by comparison with a reference composition. In some aspects, a test composition will be a specific composition

30   previously determined to be a ligand of a specific TLR. In one embodiment the test composition is a representative sample of a particular lot or batch of a specific TLR ligand. In one embodiment the test composition is a representative sample of a particular lot or batch of

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a specific TLR ligand formulated for pharmaceutical use, e.g., a sterile solution of the TLR ligand at a determined concentration or activity.

Immunostimulatory and Immunoinhibitory Nucleic Acids

5   Nucleic acids useful as reference compounds and as test compounds in the methods of the invention include single- and double-stranded natural and synthetic nucleic acids, including those with phosphodiester, stabilized, and chimeric backbones. Also encompassed are at least the following classes of nucleic acids, which are described in detail below: immunostimulatory CpG nucleic acids (CpG nucleic acids), including but not limited to types

10   A, B, and C; immunostimulatory non-CpG nucleic acids, including without limitation methylated CpG nucleic acids, T-rich nucleic acids, TG-motif nucleic acids, CpI motif nucleic acids, and poly-G nucleic acids; and immunoinhibitory nucleic acids. Nucleic acids useful as reference compounds and as test compounds in the methods of the invention also include nucleic acids with modified backbones, including "soft" and "semi-soft" oligonucleotides as

15   described herein. As will be appreciated from the descriptions below, certain of these various classes of nucleic acids can coexist in a given nucleic acid molecule.

          A "nucleic acid" as used herein with respect to test compounds and reference compounds used in the methods of the invention, shall refer to any polymer of two or more individual nucleoside or nucleotide units. Typically individual nucleoside or nucleotide units

20   will include any one or combination of deoxyribonucleosides, ribonucleosides, deoxyribonucleotides, and ribonucleotides. The individual nucleotide or nucleoside units of the nucleic acid can be naturally occurring or not naturally occurring. For example, the individual nucleotide units can include deoxyadenosine, deoxycytidine, deoxyguanosine, thymidine, and uracil. In addition to naturally occurring 2'-deoxy and 2'-hydroxyl forms,

25   individual nucleosides also include synthetic nucleosides having modified base moieties and/or modified sugar moieties, e.g., as described in Uhlmann E et al. (1990) *Chem Rev* 90:543-84. The linkages between individual nucleotide or nucleoside units can be naturally occurring or not naturally occurring. For example, the linkages can be phosphodiester, phosphorothioate, phosphorodithioate, phosphoramidate, as well as peptide linkages and other

30   covalent linkages, known in the art, suitable for joining adjacent nucleoside or nucleotide units. The linkages can also be mixed in a single polymer (e.g., a semi-soft backbone). The nucleic acid test compounds and nucleic acid reference compounds typically range in size from 3-4 units to a few tens of units, e.g., 18-40 units.

In some embodiments the nucleic acids are oligonucleotides made up of 2 to about 100 nucleotides, and more typically 4 to about 40 nucleotides. Oligonucleotides composed exclusively of deoxynucleotides are termed oligodeoxyribonucleotides or, equivalently, oligodeoxynucleotides (ODN).

5   A CpG nucleic acid is an immunostimulatory nucleic acid which contains a cytosine-guanine (CG) dinucleotide, the C residue of which is unmethylated. The effects of CpG nucleic acids on immune modulation have been described extensively in U.S. Pat. Nos. 6,194,388; 6,207,646; 6,214,806; 6,218,371; 6,239,116; and 6,339,068; and published patent applications, such as PCT/US95/01570 (WO 96/02555); PCT/US98/04703 (WO 98/40100);

10   and PCT/US99/09863 (WO 99/56755). The entire contents of each of these patents and published patent applications is hereby incorporated by reference. The entire immunostimulatory nucleic acid can be unmethylated or portions can be unmethylated, but at least the C of the 5'-CG-3' must be unmethylated. The CpG nucleic acid sequences of the invention include, without limitation, those broadly described above as well as those disclosed

15   in U.S. Pat. Nos. 6,207,646 and 6,239,116.

In one embodiment the CpG nucleic acid has a base sequence provided by 5'-TCGTCGTTTTGTCGTTTTGTCGTT-3' (SEQ ID NO:1).

In one embodiment the CpG nucleic acid has a base sequence provided by 5'-TCGTCGTTTTGACGTTTTGTCGTT-3' (SEQ ID NO:139).

20   In one embodiment the CpG nucleic acid has a base sequence provided by 5'-TCGTCGTTTTGTCGTTTTTTTCGA-3' (SEQ ID NO:140).

In one embodiment the CpG nucleic acid has a base sequence provided by 5'-TCGTCGTTTCGTCGTTTCGTCGTT-3' (SEQ ID NO:141).

In one embodiment the CpG nucleic acid has a base sequence provided by

25   5'-TCGTCGTTTCGTCGTTTGTCGTT-3' (SEQ ID NO:142).

In one embodiment the CpG nucleic acid has a base sequence provided by 5'-TCGTCGTTTTTCGGTCGTTTT-3' (SEQ ID NO:143).

In one embodiment the CpG nucleic acid has a base sequence provided by 5'-TCGTCGTTTTTCGTGCGTTTTT-3' (SEQ ID NO:144).

30   In one embodiment the CpG nucleic acid has a base sequence provided by 5'-TCGTCGTTTTCGGCGGCCGCCG-3' (SEQ ID NO:145).

In one embodiment the CpG nucleic acid has a base sequence provided by 5'-TCGTC_GTTTTAC_GGCGCC_GTGCCG-3' (SEQ ID NO:146).

The oligonucleotides described by SEQ ID NOs: 1, 139-145 are fully stabilized phosphorothioate backbone ODN. The oligonucleotide of SEQ ID NO:146 has a chimeric backbone in which all internucleoside linkages are phosphorothioate except for those indicated by "_", which are phosphodiester.

5   CpG nucleic acids have been further classified by structure and function into at least the following three types, all of which are intended to be encompassed within the methods of the instant invention: Type B CpG nucleic acids such as SEQ ID NO:1 include the earliest described CpG nucleic acids and characteristically activate B cells but do not induce or only weakly induce expression of IFN-α. Type B nucleic acids are described in U.S. Patents

10   6,194,388; 6,207,646; 6,214,806; 6,218,371; 6,239,116; and 6,339,068. Type A CpG nucleic acids, described in published international application PCT/US00/26527 (WO 01/22990), incorporate a CpG motif, include a hybrid phosphodiester/phosphorothioate backbone, and characteristically induce plasmacytoid dendritic cells to express large amounts of IFN-α but do not activate or only weakly activate B cells. Type C oligonucleotides incorporate a CpG,

15   include a chimeric backbone, include a GC-rich palindromic or nearly-palindromic region, and are capable of both activating B cells and inducing expression of IFN-α. These have been described, for example, in copending U.S. Pat. application Ser. No. 10/224,523, filed August 19, 2002. Exemplary sequences of A, B and C class nucleic acids are described in the afore-mentioned references, patents and patent applications, the entire contents of which are

20   hereby incorporated by reference herein.

        In other embodiments of the invention, a non-CpG nucleic acid is used. A non-CpG nucleic acid is an immunostimulatory nucleic acid which either does not have a CpG motif in its sequence, or has a CpG motif which contains a methylated C residue. In some instances, the non-CpG nucleic acid may still be immunostimulatory by virtue of its having other

25   immunostimulatory motifs such as those described herein and known in the art. In one embodiment the non-CpG nucleic acid is a methylated CpG nucleic acid. In some instances the non-CpG nucleic acid is still immunostimulatory despite methylation of the C of the CpG motif, even without having another non-CpG immunostimulatory motif described herein and known in the art.

30   In one embodiment the non-CpG nucleic acid is a methylated CpG nucleic acid having a base sequence provided by 5'-TZGTZGTTTTGTZGTTTTGTZGTT-3' (SEQ ID NO:147), wherein Z represents 5-methylcytosine.

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In one embodiment the non-CpG nucleic acid is a methylated CpG nucleic acid having a base sequence provided by 5'-TZGTZGZTGTZTZZGZTTZTTZTTGZZ-3' (SEQ ID NO:148), wherein Z represents 5-methylcytosine.

In one embodiment the non-CpG nucleic acid is a methylated CpG nucleic acid having a base sequence provided by 5'-GZGTTTGZTZTTZTTZTTGZG-3' (SEQ ID NO:149), wherein Z represents 5-methylcytosine.

In one embodiment the non-CpG nucleic acid is a methylated CpG nucleic acid having a base sequence provided by 5'-GZZZAAGZTGGZATZZGTZA-3' (SEQ ID NO:150), wherein Z represents 5-methylcytosine.

Non-CpG nucleic acids include T-rich immunostimulatory nucleic acids. The T-rich immunostimulatory nucleic acids include those disclosed in published PCT patent application PCT/US00/26383 (WO 01/22972), the entire contents of which are incorporated herein by reference. In some embodiments, T-rich nucleic acids 24 bases in length are used. A T-rich nucleic acid is a nucleic acid which includes at least one poly T sequence and/or which has a nucleotide composition of greater than 25% T nucleotide residues. A nucleic acid having a poly-T sequence includes at least four Ts in a row, such as 5'-TTTT-3'. In some embodiments the T-rich nucleic acid includes more than one poly T sequence. In important embodiments, the T-rich nucleic acid may have 2, 3, 4, or more poly T sequences, such as SEQ ID NO:1.

Non-CpG nucleic acids also include poly-G immunostimulatory nucleic acids. A variety of references describe the immunostimulatory properties of poly-G nucleic acids. Pisetsky DS et al. (1993) *Mol Biol Reports* 18:217-221; Krieger M et al. (1994) *Ann Rev Biochem* 63:601-637; Macaya RF et al. (1993) *Proc Natl Acad Sci USA* 90:3745-3749; Wyatt JR et al. (1994) *Proc Natl Acad Sci USA* 91:1356-1360; Rando and Hogan, 1998, In Applied Antisense Oligonucleotide Technology, Krieg and Stein, eds., pp. 335-352; Kimura Y et al. (1994) *J Biochem (Tokyo)* 116:991-994.

The immunostimulatory nucleic acids of the invention can also be those which do not possess CpG, methylated CpG, T-rich, or poly-G motifs.

Exemplary immunostimulatory nucleic acid sequences include but are not limited to those immunostimulatory sequences described and listed in U.S. Non-Provisional Pat. Application No. 09/669,187, filed on September 25, 2000; and in corresponding published PCT patent application PCT/US00/26383 (WO 01/22972).

Immunoinhibitory nucleic acids have been described in Lenert P et al. (2001) *Antisense Nucleic Acid Drug Dev* 11:247-56 and in Stunz L et al. (2002) *Eur J Immunol*

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32:1212-22. These inhibitory phosphorothioate ODN (S-ODN) differ from stimulatory S-ODN by having 2-3 G substitutions in the central motif. As inhibitory S-ODN did not directly interfere with the NF-κB DNA binding but prevented CpG-induced NF-κB nuclear translocation of p50, p65, and c-Rel and blocked p105, IκBα, and IκBβ degradation, Lenert et

5   al. suggested that the putative target of immunoinhibitory ODN would lie upstream of inhibitory kinase (IKK) activation. Stunz et al. reported that replacing GCGTT or ACGTT with GCGGG or ACGGG converted a stimulatory 15-mer ODN into an inhibitory ODN. All inhibitory ODN had three consecutive G, and a fourth G increased inhibitory activity, but a deazaguanosine substitution to prevent planar stacking did not affect activity. Inhibitory

10   ODN blocked apoptosis protection and cell-cycle entry induced by stimulatory ODN, but not that induced by lipopolysaccharide, anti-CD40 or anti-IgM+IL-4. ODN-driven up-regulation of cyclin D(2), c-Myc, c-Fos, c-Jun and Bcl(XL) and down-regulation of cyclin kinase inhibitor p27(kip1) were all blocked by inhibitory ODN. Stunz et al. also reported that interference with uptake of stimulatory ODN did not account for the inhibitory effects of the

15   immunoinhibitory nucleic acids.

In one embodiment the immunoinhibitory nucleic acid has a base sequence provided by 5'-TCCTGGCGGGGAAGT-3' (SEQ ID NO:151).

Immunoinhibitory nucleic acids have also been described in U.S. Pat. No. 6,194,388, issued to Krieg et al. The immunoinhibitory oligonucleotides disclosed by Krieg et al. are

20   oligonucleotides with GCG trinucleotides at or near the ends of the oligonucleotide and are represented by the formula 5' GCGX$_n$GCG 3' in which X is a nucleotide and n is an integer between 0 and 50.

The nucleic acids used as either test or reference compounds can be double-stranded or single-stranded. They can be deoxyribonucleotide (DNA) or ribonucleotide (RNA)

25   molecules. Generally, double-stranded molecules are more stable in vivo, while single-stranded molecules have increased immune activity. Thus in some the nucleic acid is single-stranded and in other embodiments the nucleic acid is double-stranded. In certain embodiments, while the nucleic acid is single-stranded, it is capable of forming secondary and tertiary structures (e.g., by folding back on itself, or by hybridizing with itself either

30   throughout its entirety or at select segments along its length). Accordingly, while the primary structure of such a nucleic acid may be single-stranded, its higher order structures may be double- or triple-stranded.

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For facilitating uptake into cells, the nucleic acids are preferably in the range of 6 to 100 bases in length. However, nucleic acids of any size equal to or greater than 6 nucleotides (even many kb long) are capable of inducing an immune response. Preferably the nucleic acid is in the range of between 8 and 100 and in some embodiments between 8 and 50 or 8 and 30 nucleotides in size.

The terms "nucleic acid" and "oligonucleotide" are used interchangeably to mean multiple nucleotides (i.e., molecules comprising a sugar (e.g., ribose or deoxyribose) linked to a phosphate group and to an exchangeable organic base, which is either a substituted pyrimidine (e.g., cytosine (C), thymine (T) or uracil (U)) or a substituted purine (e.g., adenine (A) or guanine (G)). As used herein, the terms "nucleic acid" and "oligonucleotide" refer to oligoribonucleotides as well as oligodeoxyribonucleotides. The terms "nucleic acid" and "oligonucleotide" shall also include polynucleosides (i.e., a polynucleotide minus the phosphate) and any other organic base containing polymer. Nucleic acid molecules can be obtained from existing nucleic acid sources (e.g., genomic or cDNA), but are preferably synthetic (e.g., produced by nucleic acid synthesis).

The terms "nucleic acid" and "oligonucleotide" also encompass nucleic acids or oligonucleotides with substitutions or modifications, such as in the bases and/or sugars. For example, they include nucleic acids having backbone sugars that are covalently attached to low molecular weight organic groups other than a hydroxyl group at the 2' position and other than a phosphate group or hydroxy group at the 5' position. Thus modified nucleic acids may include a 2'-O-alkylated ribose group. In addition, modified nucleic acids may include sugars such as arabinose or 2'-fluoroarabinose instead of ribose. Thus the nucleic acids may be heterogeneous in backbone composition thereby containing any possible combination of polymer units linked together such as peptide-nucleic acids (which have an amino acid backbone with nucleic acid bases). Other examples are described in more detail below.

The immunostimulatory and immunoinhibitory nucleic acids can encompass various chemical modifications and substitutions, in comparison to natural RNA and DNA, involving a phosphodiester internucleoside bridge, a β-D-ribose unit and/or a natural nucleoside base (adenine, guanine, cytosine, thymine, uracil). Examples of chemical modifications are known to the skilled person and are described, for example, in Uhlmann E et al. (1990) *Chem Rev* 90:543; "Protocols for Oligonucleotides and Analogs" Synthesis and Properties & Synthesis and Analytical Techniques, S. Agrawal, Ed, Humana Press, Totowa, USA 1993; Crooke ST et al. (1996) *Annu Rev Pharmacol Toxicol* 36:107-129; and Hunziker J et al. (1995) *Mod Synth*

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Methods 7:331-417. An oligonucleotide according to the invention may have one or more
modifications, wherein each modification is located at a particular phosphodiester
internucleoside bridge and/or at a particular β-D-ribose unit and/or at a particular natural
nucleoside base position in comparison to an oligonucleotide of the same sequence which is
5   composed of natural DNA or RNA.

For example, the oligonucleotides may comprise one or more modifications and
wherein each modification is independently selected from:

a)   the replacement of a phosphodiester internucleoside bridge located at the 3' and/or the
5' end of a nucleoside by a modified internucleoside bridge,

10   b)   the replacement of phosphodiester bridge located at the 3' and/or the 5' end of a
nucleoside by a dephospho bridge,

c)   the replacement of a sugar phosphate unit from the sugar phosphate backbone by
another unit,

d)   the replacement of a β-D-ribose unit by a modified sugar unit, and

15   e)   the replacement of a natural nucleoside base by a modified nucleoside base.

More detailed examples for the chemical modification of an oligonucleotide are as
follows.

The oligonucleotides may include modified internucleotide linkages, such as those
described in (a) or (b) above. These modified linkages may be partially resistant to

20   degradation (e.g., are stabilized). A "stabilized oligonucleotide molecule" shall mean an
oligonucleotide that is relatively resistant to *in vivo* degradation (e.g., via an exo- or endo-
nuclease) resulting from such modifications. Oligonucleotides having phosphorothioate
linkages, in some embodiments, may provide maximal activity and protect the oligonucleotide
from degradation by intracellular exo- and endo-nucleases.

25   A phosphodiester internucleoside bridge located at the 3' and/or the 5' end of a
nucleoside can be replaced by a modified internucleoside bridge, wherein the modified
internucleoside bridge is for example selected from phosphorothioate, phosphorodithioate,
NR^1R^2-phosphoramidate, boranophosphate, α-hydroxybenzyl phosphonate, phosphate-(C_1-
C_{21})-O-alkyl ester, phosphate-[(C_6-C_{12})aryl-(C_1-C_{21})-O-alkyl]ester, (C_1-C_8)alkylphosphonate

30   and/or (C_6-C_{12})arylphosphonate bridges, (C_7-C_{12})-α-hydroxymethyl-aryl (e.g., disclosed in
WO 95/01363), wherein (C_6-C_{12})aryl, (C_6-C_{20})aryl and (C_6-C_{14})aryl are optionally substituted
by halogen, alkyl, alkoxy, nitro, cyano, and where R^1 and R^2 are, independently of each other,
hydrogen, (C_1-C_{18})-alkyl, (C_6-C_{20})-aryl, (C_6-C_{14})-aryl-(C_1-C_8)-alkyl, preferably hydrogen,

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(C_1-C_8)-alkyl, preferably (C_1-C_4)-alkyl and/or methoxyethyl, or R^1 and R^2 form, together with the nitrogen atom carrying them, a 5-6-membered heterocyclic ring which can additionally contain a further heteroatom from the group O, S and N.

5   The replacement of a phosphodiester bridge located at the 3' and/or the 5' end of a nucleoside by a dephospho bridge (dephospho bridges are described, for example, in Uhlmann E and Peyman A in "Methods in Molecular Biology", Vol. 20, "Protocols for Oligonucleotides and Analogs", S. Agrawal, Ed., Humana Press, Totowa, 1993, Chapter 16, pp. 355 ff), wherein a dephospho bridge is for example selected from the dephospho bridges formacetal, 3'-thioformacetal, methylhydroxylamine, oxime, methylenedimethyl-hydrazo,

10   dimethylenesulfone and/or silyl groups.

A sugar phosphate unit (i.e., a β-D-ribose and phosphodiester internucleoside bridge together forming a sugar phosphate unit) from the sugar phosphate backbone (i.e., a sugar phosphate backbone is composed of sugar phosphate units) can be replaced by another unit, wherein the other unit is for example suitable to build up a "morpholino-derivative" oligomer

15   (as described, for example, in Stirchak EP et al. (1989) *Nucleic Acids Res* 17:6129-41), that is, e.g., the replacement by a morpholino-derivative unit; or to build up a polyamide nucleic acid ("PNA"; as described for example, in Nielsen PE et al. (1994) *Bioconjug Chem* 5:3-7), that is, e.g., the replacement by a PNA backbone unit, e.g., by 2-aminoethylglycine. The oligonucleotide may have other carbohydrate backbone modifications and replacements, such

20   as peptide nucleic acids with phosphate groups (PHONA), locked nucleic acids (LNA), and oligonucleotides having backbone sections with alkyl linkers or amino linkers. The alkyl linker may be branched or unbranched, substituted or unsubstituted, and chirally pure or a racemic mixture.

A β-ribose unit or a β-D-2'-deoxyribose unit can be replaced by a modified sugar unit,

25   wherein the modified sugar unit is for example selected from β-D-ribose, α-D-2'-deoxyribose, L-2'-deoxyribose, 2'-F-2'-deoxyribose, 2'-F-arabinose, 2'-O-(C_1-C_6)alkyl-ribose, preferably 2'-O-(C_1-C_6)alkyl-ribose is 2'-O-methylribose, 2'-O-(C_2-C_6)alkenyl-ribose, 2'-[O-(C_1-C_6)alkyl-O-(C_1-C_6)alkyl]-ribose, 2'-NH_2-2'-deoxyribose, β-D-xylo-furanose, α-arabinofuranose, 2,4-dideoxy-β-D-erythro-hexo-pyranose, and carbocyclic (described, for example, in Froehler

30   J (1992) *Am Chem Soc* 114:8320) and/or open-chain sugar analogs (described, for example, in Vandendriessche et al. (1993) *Tetrahedron* 49:7223) and/or bicyclosugar analogs (described, for example, in Tarkov M et al. (1993) *Helv Chim Acta* 76:481).

In some embodiments the sugar is 2'-O-methylribose, particularly for one or both nucleotides linked by a phosphodiester or phosphodiester-like internucleoside linkage.

In some embodiments, the nucleic acids may be soft or semi-soft nucleic acids. A soft nucleic acid is an immunostimulatory nucleic acid having a partially stabilized backbone, in which phosphodiester or phosphodiester-like internucleotide linkages occur only within and immediately adjacent to at least one internal pyrimidine -purine dinucleotide (YZ). Preferably YZ is YG, a pyrimidine-guanosine (YG) dinucleotide. The at least one internal YZ dinucleotide itself has a phosphodiester or phosphodiester-like internucleotide linkage. A phosphodiester or phosphodiester-like internucleotide linkage occurring immediately adjacent to the at least one internal YZ dinucleotide can be 5', 3', or both 5' and 3' to the at least one internal YZ dinucleotide.

In particular, phosphodiester or phosphodiester-like internucleotide linkages involve "internal dinucleotides". An internal dinucleotide in general shall mean any pair of adjacent nucleotides connected by an internucleotide linkage, in which neither nucleotide in the pair of nucleotides is a terminal nucleotide, i.e., neither nucleotide in the pair of nucleotides is a nucleotide defining the 5' or 3' end of the nucleic acid. Thus a linear nucleic acid that is n nucleotides long has a total of n-1 dinucleotides and only n-3 internal dinucleotides. Each internucleotide linkage in an internal dinucleotide is an internal internucleotide linkage. Thus a linear nucleic acid that is n nucleotides long has a total of n-1 internucleotide linkages and only n-3 internal internucleotide linkages. The strategically placed phosphodiester or phosphodiester-like internucleotide linkages, therefore, refer to phosphodiester or phosphodiester-like internucleotide linkages positioned between any pair of nucleotides in the nucleic acid sequence. In some embodiments the phosphodiester or phosphodiester-like internucleotide linkages are not positioned between either pair of nucleotides closest to the 5' or 3' end.

Preferably a phosphodiester or phosphodiester-like internucleotide linkage occurring immediately adjacent to the at least one internal YZ dinucleotide is itself an internal internucleotide linkage. Thus for a sequence N_1 YZ N_2, wherein N_1 and N_2 are each, independent of the other, any single nucleotide, the YZ dinucleotide has a phosphodiester or phosphodiester-like internucleotide linkage, and in addition (a) N_1 and Y are linked by a phosphodiester or phosphodiester-like internucleotide linkage when N_1 is an internal nucleotide, (b) Z and N_2 are linked by a phosphodiester or phosphodiester-like internucleotide linkage when N_2 is an internal nucleotide, or (c) N_1 and Y are linked by a phosphodiester or

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phosphodiester-like internucleotide linkage when N_1 is an internal nucleotide and Z and N_2 are linked by a phosphodiester or phosphodiester-like internucleotide linkage when N_2 is an internal nucleotide.

Soft nucleic acids according to the instant invention are believed to be relatively
5   susceptible to nuclease cleavage compared to completely stabilized nucleic acids. Without meaning to be bound to a particular theory or mechanism, it is believed that soft nucleic acids of the invention are cleavable to fragments with reduced or no immunostimulatory activity relative to full-length soft nucleic acids. Incorporation of at least one nuclease-sensitive internucleotide linkage, particularly near the middle of the nucleic acid, is believed to provide
10   an "off switch" which alters the pharmacokinetics of the nucleic acid so as to reduce the duration of maximal immunostimulatory activity of the nucleic acid. This can be of particular value in tissues and in clinical applications in which it is desirable to avoid injury related to chronic local inflammation or immunostimulation, e.g., the kidney.

A semi-soft nucleic acid is an immunostimulatory nucleic acid having a partially
15   stabilized backbone, in which phosphodiester or phosphodiester-like internucleotide linkages occur only within at least one internal pyrimidine-purine (YZ) dinucleotide. Semi-soft nucleic acids generally possess increased immunostimulatory potency relative to corresponding fully stabilized immunostimulatory nucleic acids. Due to the greater potency of semi-soft nucleic acids, semi-soft nucleic acids may be used, in some instances, at lower
20   effective concentations and have lower effective doses than conventional fully stabilized immunostimulatory nucleic acids in order to achieve a desired biological effect.

It is believed that the foregoing properties of semi-soft nucleic acids generally increase with increasing "dose" of phosphodiester or phosphodiester-like internucleotide linkages involving internal YZ dinucleotides. Thus it is believed, for example, that generally for a
25   given nucleic acid sequence with five internal YZ dinucleotides, an nucleic acid with five internal phosphodiester or phosphodiester-like YZ internucleotide linkages is more immunostimulatory than an nucleic acid with four internal phosphodiester or phosphodiester-like YG internucleotide linkages, which in turn is more immunostimulatory than an nucleic acid with three internal phosphodiester or phosphodiester-like YZ internucleotide linkages,
30   which in turn is more immunostimulatory than an nucleic acid with two internal phosphodiester or phosphodiester-like YZ internucleotide linkages, which in turn is more immunostimulatory than an nucleic acid with one internal phosphodiester or phosphodiester-like YZ internucleotide linkage. Importantly, inclusion of even one internal phosphodiester or

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phosphodiester-like YZ internucleotide linkage is believed to be advantageous over no internal phosphodiester or phosphodiester-like YZ internucleotide linkage. In addition to the number of phosphodiester or phosphodiester-like internucleotide linkages, the position along the length of the nucleic acid can also affect potency.

5   The soft and semi-soft nucleic acids will generally include, in addition to the phosphodiester or phosphodiester-like internucleotide linkages at preferred internal positions, 5' and 3' ends that are resistant to degradation. Such degradation-resistant ends can involve any suitable modification that results in an increased resistance against exonuclease digestion over corresponding unmodified ends. For instance, the 5' and 3' ends can be stabilized by the

10   inclusion thereof at least one phosphate modification of the backbone. In a preferred embodiment, the at least one phosphate modification of the backbone at each end is independently a phosphorothioate, phosphorodithioate, methylphosphonate, or methylphosphorothioate internucleotide linkage. In another embodiment, the degradation-resistant end includes one or more nucleotide units connected by peptide or amide linkages at

15   the 3' end.

            A phosphodiester internucleotide linkage is the type of linkage characteristic of nucleic acids found in nature. The phosphodiester internucleotide linkage includes a phosphorus atom flanked by two bridging oxygen atoms and bound also by two additional oxygen atoms, one charged and the other uncharged. Phosphodiester internucleotide linkage

20   is particularly preferred when it is important to reduce the tissue half-life of the nucleic acid.

            A phosphodiester-like internucleotide linkage is a phosphorus-containing bridging group that is chemically and/or diastereomerically similar to phosphodiester. Measures of similarity to phosphodiester include susceptibility to nuclease digestion and ability to activate RNAse H. Thus for example phosphodiester, but not phosphorothioate, nucleic acids are

25   susceptible to nuclease digestion, while both phosphodiester and phosphorothioate nucleic acids activate RNAse H. In a preferred embodiment the phosphodiester-like internucleotide linkage is boranophosphate (or equivalently, boranophosphonate) linkage. U.S. Patent No. 5,177,198; U.S. Patent No. 5,859,231; U.S. Patent No. 6,160,109; U.S. Patent No. 6,207,819; Sergueev et al., (1998) *J Am Chem Soc* 120:9417-27. In another preferred embodiment the

30   phosphodiester-like internucleotide linkage is diasteromerically pure Rp phosphorothioate. It is believed that diasteromerically pure Rp phosphorothioate is more susceptible to nuclease digestion and is better at activating RNAse H than mixed or diastereomerically pure Sp phosphorothioate. Stereoisomers of CpG nucleic acids are the subject of co-pending U.S.

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patent application 09/361,575 filed July 27, 1999, and published PCT application
PCT/US99/17100 (WO 00/06588). It is to be noted that for purposes of the instant invention,
the term "phosphodiester-like internucleotide linkage" specifically excludes
phosphorodithioate and methylphosphonate internucleotide linkages.

5   As described above the soft and semi-soft nucleic acids of the invention may have
phosphodiester like linkages between C and G. One example of a phosphodiester-like linkage
is a phosphorothioate linkage in an Rp conformation. Nucleic acid p-chirality can have
apparently opposite effects on the immune activity of a CpG nucleic acid, depending upon the
time point at which activity is measured. At an early time point of 40 minutes, the R_p but not
10   the S_P stereoisomer of phosphorothioate CpG nucleic acid induces JNK phosphorylation in
mouse spleen cells. In contrast, when assayed at a late time point of 44 hr, the S_P but not the
R_p stereoisomer is active in stimulating spleen cell proliferation. This difference in the
kinetics and bioactivity of the R_p and S_P stereoisomers does not result from any difference in
cell uptake, but rather most likely is due to two opposing biologic roles of the p-chirality.
15   First, the enhanced activity of the Rp stereoisomer compared to the Sp for stimulating
immune cells at early time points indicates that the Rp may be more effective at interacting
with the CpG receptor, TLR9, or inducing the downstream signaling pathways. On the other
hand, the faster degradation of the Rp PS-nucleic acids compared to the Sp results in a much
shorter duration of signaling, so that the Sp PS-nucleic acids appear to be more biologically
20   active when tested at later time points.

         A surprisingly strong effect is achieved by the p-chirality at the CpG dinucleotide
itself. In comparison to a stereo-random CpG nucleic acid the congener in which the single
CpG dinucleotide was linked in Rp was slightly more active, while the congener containing
an Sp linkage was nearly inactive for inducing spleen cell proliferation.

25   Nucleic acids also include substituted purines and pyrimidines such as C-5 propyne
pyrimidine and 7-deaza-7-substituted purine modified bases. Wagner RW et al. (1996) *Nat
Biotechnol* 14:840-4. Purines and pyrimidines include but are not limited to adenine,
cytosine, guanine, and thymine, and other naturally and non-naturally occurring nucleobases,
substituted and unsubstituted aromatic moieties.

30   A modified base is any base which is chemically distinct from the naturally occurring
bases typically found in DNA and RNA such as T, C, G, A, and U, but which share basic
chemical structures with these naturally occurring bases. The modified nucleoside base may
be, for example, selected from hypoxanthine, uracil, dihydrouracil, pseudouracil, 2-thiouracil,

4-thiouracil, 5-aminouracil, 5-(C_1-C_6)-alkyluracil, 5-(C_2-C_6)-alkenyluracil, 5-(C_2-C_6)-
alkynyluracil, 5-(hydroxymethyl)uracil, 5-chlorouracil, 5-fluorouracil, 5-bromouracil,
5-hydroxycytosine, 5-(C_1-C_6)-alkylcytosine, 5-(C_2-C_6)-alkenylcytosine, 5-(C_2-C_6)-
alkynylcytosine, 5-chlorocytosine, 5-fluorocytosine, 5-bromocytosine, N^2-dimethylguanine,

5   2,4-diamino-purine, 8-azapurine, a substituted 7-deazapurine, preferably
7-deaza-7-substituted and/or 7-deaza-8-substituted purine, 5-hydroxymethylcytosine, N4-
alkylcytosine, e.g., N4-ethylcytosine, 5-hydroxydeoxycytidine, 5-
hydroxymethyldeoxycytidine, N4-alkyldeoxycytidine, e.g., N4-ethyldeoxycytidine, 6-
thiodeoxyguanosine, and deoxyribonucleosides of nitropyrrole, C5-propynylpyrimidine, and

10   diaminopurine e.g., 2,6-diaminopurine, inosine, 5-methylcytosine, 2-aminopurine,
2-amino-6-chloropurine, hypoxanthine or other modifications of a natural nucleoside bases.
This list is meant to be exemplary and is not to be interpreted to be limiting.

        Modified cytosines include but are not limited to 5-substituted cytosines (e.g., 5-
methyl-cytosine, 5-fluoro-cytosine, 5-chloro-cytosine, 5-bromo-cytosine, 5-iodo-cytosine, 5-

15   hydroxy-cytosine, 5-hydroxymethyl-cytosine, 5-difluoromethyl-cytosine, and unsubstituted or
substituted 5-alkynyl-cytosine), 6-substituted cytosines, N4-substituted cytosines (e.g., N4-
ethyl-cytosine), 5-aza-cytosine, 2-mercapto-cytosine, isocytosine, pseudo-isocytosine,
cytosine analogs with condensed ring systems (e.g., N,N'-propylene cytosine or
phenoxazine), and uracil and its derivatives (e.g., 5-fluoro-uracil, 5-bromo-uracil, 5-

20   bromovinyl-uracil, 4-thio-uracil, 5-hydroxy-uracil, 5-propynyl-uracil). In another
embodiment, the cytosine base is substituted by a universal base (e.g., 3-nitropyrrole, P-base),
an aromatic ring system (e.g., fluorobenzene or difluorobenzene) or a hydrogen atom
(dSpacer).

        Modified guanines include but are not limited to 7-deazaguanine,

25   7-deaza-7-substituted guanine (such as 7-deaza-7-(C2-C6)alkynylguanine),
7-deaza-8-substituted guanine, hypoxanthine, N2-substituted guanines (e.g., N2-methyl-
guanine), 5-amino-3-methyl-3H,6H-thiazolo[4,5-d]pyrimidine-2,7-dione, 2,6-diaminopurine,
2-aminopurine, purine, indole, adenine, substituted adenines (e.g., N6-methyl-adenine, 8-oxo-
adenine) 8-substituted guanine (e.g., 8-hydroxyguanine and 8-bromoguanine), and

30   6-thioguanine. In another embodiment, the guanine base is substituted by a universal base
(e.g., 4-methyl-indole, 5-nitro-indole, and K-base), an aromatic ring system (e.g.,
benzimidazole or dichloro-benzimidazole, 1-methyl-1H-[1,2,4]triazole-3-carboxylic acid
amide) or a hydrogen atom (dSpacer).

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For use in the instant invention, the oligonucleotide reference compounds and test compounds can be synthesized *de novo* using any of a number of procedures well known in the art, for example, the β-cyanoethyl phosphoramidite method (Beaucage SL et al. (1981) *Tetrahedron Lett* 22:1859), or the nucleoside H-phosphonate method (Garegg et al. (1986)

5   *Tetrahedron Lett* 27:4051-4; Froehler BC et al. (1986) *Nucleic Acids Res* 14:5399-407; Garegg et al (1986) *Tetrahedron Lett* 27:4055-8; Gaffney et al. (1988) *Tetrahedron Lett* 29:2619-22). These chemistries can be performed by a variety of automated nucleic acid synthesizers available in the market. These oligonucleotides are referred to as synthetic oligonucleotides. An isolated oligonucleotide generally refers to an oligonucleotide which is

10   separated from components which it is normally associated with in nature. As an example, an isolated oligonucleotide may be one which is separated from a cell, from a nucleus, from mitochondria or from chromatin.

Modified backbones such as phosphorothioates can be synthesized using automated techniques employing either phosphoramidate or H-phosphonate chemistries. Aryl-and

15   alkyl-phosphonates can be made, e.g., as described in U.S. Pat. No. 4,469,863; and alkylphosphotriesters (in which the charged oxygen moiety is alkylated as described in U.S. Pat. No. 5,023,243 and European Pat. No. 092,574) can be prepared by automated solid phase synthesis using commercially available reagents. Methods for making other DNA backbone modifications and substitutions have been described (e.g., Uhlmann E et al. (1990) *Chem Rev*

20   90:544; Goodchild J (1990) *Bioconjugate Chem* 1:165).

TLR expression

The cell lines can be used in their native state without any modification. For example, in the case of the RPMI 8226 cell line, it can be used to identify compounds that signal

25   through at least TLR9 and/or TLR7. In other instances, however, the cell line can be modified to express a TLR that it does not naturally express. In still other instances, the cell to be used in the screening method may express one or more endogenous TLR and yet still be manipulated to express an additional TLR different from those it endogenously expresses. The cell may also be manipulated in order to increase or decrease the level of TLR that it

30   endogenously expresses. The cells may be stably or transiently transfected.

A cell that does not naturally express a protein or polypeptide, but is genetically manipulated to do so is referred to as ectopically expressing the protein or polypeptide.

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The basic screening method remains the same regardless of which TLR is expressed by the cell. However, the reference compound and the readout may vary depending upon the TLR(s) expressed. In the most simple aspect, the screening method is used to identify a compound that signals through a TLR such as for example TLR9. In this case, the positive

5   reference compound may be an immunostimulatory compound already known to act through TLR9 (e.g., CpG nucleic acid).

The methods of the invention involve, in part, contacting a functional TLR with a test composition. A functional TLR is a full-length TLR protein or a fragment thereof capable of inducing or inhibiting a signal in response to interaction with its ligand. Generally the

10 functional TLR will include at least a TLR ligand-binding fragment of the extracellular domain of the full-length TLR and at least a fragment of a TIR domain capable of interacting with another Toll homology domain-containing polypeptide, e.g., MyD88. In various embodiments the functional TLR is a full-length TLR selected from TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, and TLR10.

15 To date, there are eleven TLRs known. Nucleic acid and amino acid sequences for ten currently known human TLRs are available from public databases such as GenBank. Similarly, nucleic acid and amino acid sequences for various TLRs from numerous non-human species are also available from public databases including GenBank. For example, nucleic acid and amino acid sequences for human TLR9 (hTLR9) can be found as GenBank

20 accession numbers AF245704 (coding region spanning nucleotides 145-3243) (SEQ ID NO: 60) and AAF78037 (SEQ ID NO: 62), respectively. Nucleic acid and amino acid sequences for murine TLR9 (mTLR9) can be found as GenBank accession numbers AF348140 (coding region spanning nucleotides 40-3138) (SEQ ID NO: 68) and AAK29625 (SEQ ID NO: 72), respectively.

25 Nucleic acid and amino acid sequences for human TLR8 (hTLR8) can be found as GenBank accession numbers AF245703 (coding region spanning nucleotides 49-3174) (SEQ ID NO: 46) and AAF78036 (SEQ ID NO: 50), respectively. Nucleic acid and amino acid sequences for murine TLR8 (mTLR8) can be found as GenBank accession numbers AY035890 (coding region spanning nucleotides 59-3157) (SEQ ID NO: 55) and AAK62677

30 (SEQ ID NO: 57), respectively.

Nucleic acid and amino acid sequences for human TLR7 (hTLR7) can be found as GenBank accession numbers AF240467 (coding region spanning nucleotides 135-3285) (SEQ ID NO: 31) and AAF60188 (SEQ ID NO: 34), respectively. Nucleic acid and amino acid

sequences for murine TLR7 (mTLR7) can be found as GenBank accession numbers AY035889 (coding region spanning nucleotides 49-3201) (SEQ ID NO: 38) and AAK62676 (SEQ ID NO: 41), respectively.

Nucleic acid and amino acid sequences for human TLR3 (hTLR3) can be found as

5   GenBank accession numbers NM_003265 (coding region spanning nucleotides 102-2816) (SEQ ID NO: 7) and NP_003256 (SEQ ID NO: 8), respectively. Nucleic acid and amino acid sequences for murine TLR3 (hTLR3) can be found as GenBank accession numbers AF355152 (coding region spanning nucleotides 44-2761) (SEQ ID NO: 9) and AAK26117 (SEQ ID NO: 10), respectively.

10   Nucleic acid and amino acid sequences for human TLR1 (hTLR1) can be found as GenBank accession numbers NM_003263 and NP_003254, respectively. Nucleic acid and amino acid sequences for murine TLR1 (mTLR1) can be found as GenBank accession numbers NM_030682 and NP_109607, respectively.

The functional TLR also is not limited to native TLR polypeptides. As used herein, a

15   native TLR is one that is naturally occurring. The TLR may be a non-native (or non-naturally occurring TLR). An example is a chimeric TLR having an extracellular domain and the cytoplasmic domain derived from TLRs from different species. Such chimeric TLR polypeptides can include, for example, a human TLR extracellular domain and a murine TLR cytoplasmic domain. In alternative embodiments, such chimeric TLR polypeptides can

20   include chimerae created with different TLR splice variants or allotypes.

TLR Signaling Pathways

The screening methods provided by the invention measure TLR signaling activity. TLR signaling activity is activity that results from interaction of a TLR with a TLR ligand.

25   TLR signaling can be measured in a number of ways including but not limited to interaction between a TLR and a protein or factor (such as an adaptor protein), interaction between downstream proteins or factors (such as an adaptor protein) with each other, activation of nuclear factors such as transcription factors or transcription complexes, up- or down-regulation of genes, phosphorylation or dephosphorylation of proteins or factors in the

30   signaling cascade, expression, production and/or secretion of cytokines and/or chemokines, changes in cell cycle status, up- or down-regulation of cell surface marker expression, and the like. Those of ordinary skill in the art are familiar with assays for measuring these latter

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events including but not limited to gel shift assays, immunoprecipitations, phosphorylation . status analysis of proteins, Northern analysis, RT-PCR analysis, etc.

5   The following is an exemplary TLR signaling pathway or cascade. It is to be understood that this is meant to be illustrative and that different factors may be involved in the signaling of particular TLR. One TLR signaling pathway is known to use the cytoplasmic Toll/IL-1 receptor (TIR) homology domain, present in all TLRs. This domain interacts (e.g., binds to) and thereby transduces a signal to a similar domain on an adapter protein (e.g., MyD88). This type of interaction is referred to as a like:like interaction of TIR domains. This interaction is followed by an another interaction between the adapter protein and a

10   kinase, through their respective "death domains". In the case of at least TLR4 signaling, the kinase then interacts with tumor necrosis factor (TNF) receptor-associated factor-6 (TRAF6). Medzhitov R et al., *Mol Cell* 2:253 (1998); Kopp EB et al., *Curr Opin Immunol* 11:15 (1999). After TRAF6, two sequential kinase activation steps lead to phosphorylation of the inhibitory protein I kappa B and its dissociation from NF-κB. The first kinase is a mitogen-activated

15   kinase kinase kinase (MAPKKK) known as NIK, for NF-κB-inducing kinase. The target of this kinase is another kinase made up of two chains, called I kappa B kinase α (IKK α) and I kappa B kinase β (IKK β), that together form a heterodimer of IKKα:IKKβ, which phosphorylates I kappa B. NF-κB translocates to the nucleus to activate genes with kappa B binding sites in their promoters and enhancers such as the genes encoding IL-6, IL-8, the p40

20   subunit of IL-12, and the costimulatory molecule CD86. The signaling mechanisms of TLRs are not limited to this pathway; other signaling pathways exist and can be used in the screening readouts of the methods provided herein.

     The screening assays employ a number of readouts (or parameters). The readouts can be native readouts. A native readout is one that does not rely on introduction of a reporter

25   construct into the cell of interest. The readouts can be artificial. An artificial readout is one that relies on introduction of a reporter construct into the cell of interest. Examples of both are provided herein. In still other embodiments, a given assay may measure one or more native readouts and one or more artificial readouts. Each readout whether native or artificial is related to signaling pathways that ensue after TLR engagement with a ligand.

30   Each cell line described herein will be associated with a particular set of native readouts which the invention seeks to determine in the screening assays provided. As an example, the response of the RPMI 8226 cell line to an immunomodulatory molecule can be assessed in terms of native readouts such as CD71 expression, CD86 expression, HLA-DR

expression, IL-8 expression, IL-8 production, IL-8 secretion, IL-10 expression, IL-10

production, IL-10 secretion, IP-10 expression, IP-10 production, IP-10 secretion, TNF-α

expression, TNF-α production and TNF-α secretion. RAMOS response can be assessed, inter

alia, by CD80 cell surface expression. Raji response can be assessed, inter alia, by IL-6

5   secretion.

As described in greater detail herein, the cell line can be used in an unmodified form.

In one respect, an unmodified cell line will naturally respond to a TLR ligand through a native

readout system. For example, an RPMI 8226 cell exposed to an immunostimulatory TLR

ligand may increase expression of IP-10 from the native gene locus. Alternatively, the cell

10   line may be modified to contain a reporter construct that acts as a surrogate for the IP-10 gene

locus. For example, the reporter construct may contain the TLR responsive promoter

elements that are naturally found in the native IP-10 locus operably linked to a reporter coding

sequence that encodes a gene product that is detectable and quantifiable. The structure and

variability of suitable reporter constructs will be discussed in greater detail herein.

15   Readouts typically include the induction of a gene under control of a specific promoter

such as a NF-κB promoter. The gene under the control of the NF-κB promoter can be a gene

which naturally includes an NF-κB promoter or it can be a gene in a construct in which an

NF-κB promoter has been inserted. Endogenous genes and transfected constructs which

include the NF-κB promoter include but are not limited to IL-8, IL-12 p40, NF-κB-luc, IL-12

20   p40-luc, and TNF-luc.

Increases in cytokine levels can result from increased production, increased stability,

increased secretion, or any combination of the forgoing, of the cytokine in response to the

TLR-mediated signaling. Cytokines generally include, without limitation, IL-1, IL-2, IL-3,

IL-4, IL-5, IL-6, IL-7, IL-10, IL-11, IL-12, IL-13, IL-15, IL-18, IFN-α, IFN-β, IFN-γ, TNF-α,

25   GM-CSF, G-CSF, M-CSF. Th1 cytokines include but are not limited to IL-2, IFN-γ, and IL-

12. Th2 cytokines include but are not limited to IL-4, IL-5, and IL-10.

Increases in chemokine levels can result from increased production, increased

stability, increased secretion, or any combination of the forgoing, of the chemokine in

response to the TLR-mediated signaling. Chemokines of particular significance in the

30   invention include but are not limited to CCL5 (RANTES), CXCL9 (Mig), CXCL10 (IP-10),

CXCL11 (I-TAC), IL-8, and MCP-1.

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TLR signaling activity can also be measured by phosphorylation, such as total cellular phosphorylation or phosphorylation of specific factors such as but not limited to IRAK, ERK, MyD88, TRAF6, p38, NF-κB subunits, c-Jun and c-Fos.

TLR signaling activity can be measured by changes in gene expression. The

5   expression of CD71, CD86, CD80, CD69, CD54, HLA-DR, HLA class I, IL-6, IL-8, IL-10, IP-9, IP-10, IFN-α, TNF-α, and the like can be assessed as a measure of TLR signaling activity. Gene expression analysis may be performed using microarray techniques.

TLR signaling activity can also be measured by cell proliferation status or changes thereto.

10   TLR signaling activity can also be measured by cell surface marker expression such as the cell surface expression of markers such as but not limited to CD71, CD86, HLA-DR, CD80, HLA class I, CD54 and CD69.

TLR signaling activity can also be measured by antibody secretion such as but not limited to IgM secretion.

15

Reporter and Expression Constructs

The cells can be manipulated by the introduction of expression and/or reporter constructs. The expression constructs preferably comprise a TLR coding sequence, as described above. The reporter constructs can be used as surrogate measures of native TLR

20   signaling activity. These reporter constructs are intended to substitute for the "native" readouts capable with the cell line. In order to act as substitutes, the reporter constructs include a promoter element derived from a gene known to be modulated following TLR engagement with a TLR ligand. The reporter construct further includes a coding sequence linked to the promoter. The coding sequence is usually that of a reporter (i.e., a protein that is

25   detectable or quantifiable).

The reporter construct generally includes a promoter, a coding sequence and a polyadenylation signal. These nucleic acids shall include, as necessary, 5' non-transcribing and 5' non-translating sequences involved with the initiation of transcription and translation, respectively, such as a TATA box, capping sequence, CAAT sequence, in addition to

30   promoter elements that are responsive to TLR signaling. The nucleic acid constructs may optionally include enhancer sequences or upstream activator sequences as desired.

The promoter in the reporter construct will include a TLR responsive promoter element, and will therefore be regarded as a TLR responsive promoter. As used herein, a

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TLR responsive promoter is a promoter having an activity that is modulated (i.e., either activated or inhibited) by signaling through a TLR (e.g., by TLR interaction with its ligand). In order to be modulated by TLR signaling, the promoter contains sites that are bound by transcription factors modulated by TLR signaling. The factors may be activated or inhibited

5   by TLR signaling. Activation of the transcription factor includes increases in the activity of the transcription factor per se, increases in its ability to interact with other factors or with DNA that serve to increase its activity, and increases in its transcription and translation (i.e., increased mRNA and protein levels of the transcription factor). Conversely, inhibition of the transcription factor includes decreases in the activity of the transcription factor per se,

10   decreases in its ability to interact with other factors or with DNA that serve to decrease its activity, and decreases in its transcription and translation (i.e., decreased mRNA and protein levels of the transcription factor). The effect on the transcription factor is usually the downstream result of other interactions in the signaling pathway. The expression of coding sequences linked to such promoters will therefore be modulated by TLR signaling events, and

15   it is the level of expression of these coding sequences that can be used as a readout of TLR signaling in the screening methods provided herein.

The TLR responsive promoter may comprise a transcription factor binding site selected from the group consisting of a NF-κB binding site, an AP-1 binding site, a CRE, a SRE, an interferon-stimulated response element (ISRE), a GAS, an ATF2 binding site, an

20   IRF3 binding site, an IRF7 binding site, an NFAT binding site, a p53 binding site, an SRF binding site, and a TARE, among others. These binding sites and their sequences are known in the art. Below is a exemplary list of these sequences.
W = A or T, R = A or G, Y = C or T

25   NF-κB Binding site:

        Consensus p50 subunit
        5' GGGGATYCCC 3' (SEQ ID NO:90)

30   Consensus p65 subunit
        5' GGGRNTTTCC 3' (SEQ ID NO:91)

        Example of p65 subunit binding site
        5' AGT TGA GGG GAC TTT CCC AGG C 3' (SEQ ID NO:92)

35
     CREB Binding site:
        5'AGA GAT TGC CTG ACG TCA GAG AGC TAG 3' (SEQ ID NO:93)

AP-1 Binding site:
                    5'- CGC TTG ATG AGT CAG CCG GAA –3' (SEQ ID NO:94)
                    5'- CGC ATG AGT CAG ACA –3' (SEQ ID NO:95)

5   ISRE :

                    5'- TGCAGAAGTGAAACTGAGG-3' (SEQ ID NO:96)
                    5'- AGAACGAAACA-3' (SEQ ID NO:97)
                    5'- GAGAAGTGAAAGTGG-3' (SEQ ID NO:98)
                    5'- TAAGAACATGAAACTGAA-3' (SEQ ID NO:99)
10   5'- ATGAAACTGAAAGTA-3' (SEQ ID NO:100)
                    5'- TGAAAACCGAAAGCGC-3' (SEQ ID NO:101)
                    5'- AGAAATGGAAAGT-3' (SEQ ID NO:102)

      SRE
15   5'- TCACCCCAC-3' (SEQ ID NO:103)
                    5'- CTCACCCCAC-3' (SEQ ID NO:104)
                    5'- GCCACCCTAC-3' (SEQ ID NO:105)

      NFAT:
20   5'- TATGAAACAGTTTTTCC –3' (SEQ ID NO:106)
                    5'- AGGAAACTC –3' (SEQ ID NO:107)
                    5'- ARGARATTCC –3' (SEQ ID NO:108)
                    5'- CCAGTTGAGCCAGAGA –3' (SEQ ID NO:109)

25   GAS:

                    5'- CTTTCAGTTTCATATTACTCTAAATCCATT –3' (SEQ ID NO:110)

      p53 Binding Site :

30   p53 Consensus site:
                    5'- RRRCWWGYYY –3' (SEQ ID NO:111)

                    Examples of p53 binding sites:
                    5'- AGGCATGCCT –3' (SEQ ID NO:112)
                    5'- GGGCTTGCCC –3' (SEQ ID NO:113)
35   5'- GGGCTTGCTT –3' (SEQ ID NO:114)
                    5'- GCCTGGACTTGCC –3' (SEQ ID NO:115)
                    5'- GGACATGCCCGGGCATGTCC –3' (SEQ ID NO:116)
                    5'- GTAGCATTAGCCCAGACATGTCC –3' (SEQ ID NO:117)
40
      TARE (TNF-α response element):
      e.g. from the COL1A1 promoter
                    5'GAGGTATGCAGACAAGAGTCAGAGTTTCCCCTTGAA 3' (SEQ ID
      NO:118)
45
      SRF
                    5'- CCWWWWWWGG –3' (SEQ ID NO:119)
                    5'- CCAAATAAGGC –3' (SEQ ID NO:120)

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The TLR responsive promoter element can be derived from the promoter of a naturally occurring (i.e., an endogenous) gene that is activated or inhibited by TLR signaling (such as the IL-6 gene, the IL-8 gene, the IL-10 gene, the IL-12 p40 gene, the IP-9 gene, the IP-10 gene, the type 1 IFN gene, the IFN-α4 gene, the IFN-β gene, the TNF-α gene, the TNF-β

5   gene, the RANTES gene, the ITAC gene, the IGFBP4 gene, the CD54 gene, the CD69 gene, the CD71 gene, the CD80 gene, the CD86 gene, the HLA-DR gene, the HLA class I gene, and the like). The afore-mentioned genes are genes that are known to be activated in response to TLR interaction with its ligand.

Suitable promoter regions are described in the Examples. Briefly, the upstream (5') –

10   620 to +50 promoter region of IFN-α4 or the upstream (5') –140 to +9 promoter region of IFN-α1 can be used. In one embodiment, the IFN-α4 sequence is cloned into the SmaI site of the pGL3-Basic Vector (Promega) resulting in an expression vector that includes a luciferase gene under the control of the upstream (5') promoter region of IFN-α4.

The promoter can also be the upstream (5') –280 to +20 promoter region of IFN-β.

15   The promoter can also be the upstream (5') –397 to +5 promoter region of RANTES. In one embodiment, the RANTES promoter sequence is cloned into the NheI site (filled in with Klenow) of the pGL3-Basic Vector (Promega) resulting in an expression vector that includes a luciferase gene under the control of the upstream (5') –397 to +5 promoter region of RANTES.

20   The promoter can also be the upstream truncated (-250 to +30) and full length (-860 to +30) promoter regions derived from human IL-12 p40 genomic DNA. In one embodiment, the truncated IL-12 p40 promoter was cloned as a KpnI-XhoI insert into pβgal-Basic (Promega) resulting in an expression vector that includes a β gal gene under the control of the upstream (5') –250 to +30 promoter region of human IL-12 p40. In another embodiment, the

25   full length IL-12 p40 promoter was cloned as a KpnI-XhoI insert into pβgal-Basic (Promega) resulting in an expression vector that includes a β gal gene under the control of the upstream (5') –751 to +30 promoter region of human IL-12 p40. In another embodiment, the truncated –250 to +30 promoter region of human IL-12 p40 was cloned into the pGL3-Basic Vector (Promega) resulting in an expression vector that includes a luciferase gene under the control

30   of the upstream (5') –250 to +30 promoter region of human IL-12 p40. In yet another embodiment, the full length IL-12 p40 promoter of human IL-12 p40 was cloned into the

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pGL3-Basic Vector (Promega) resulting in an expression vector that includes a luciferase gene under the control of the upstream (5') −751 to +30 promoter region of human IL-12 p40.

The promoter can also be the upstream (5') -288 to +7 promoter region derived from human IL-6 genomic DNA. The promoter can also be derived from the full-length promoter

5   region of the IL-6 gene from −1174 to + 7 (Accession No M22111, SEQ ID NO:129).

The promoter can also be the upstream (5') -734 to +44 or the upstream (5') -162 to +44 promoter region derived from human IL-8 genomic DNA. Mukaida N et al. (1989) *J Immunol* 143:1366-71.

The promoter can also be derived from the -615 to +30 promoter region of human

10   TNF-α.

The promoter can also be derived from a promoter region of human TNF-β.

The promoter can also be derived from the −875 to +97 promoter region of human IP-

10.

The promoter can also be derived from the −219 to +114 promoter region of human

15   CXCL11 (IP9). The promoter can also be derived from the full length (-934 to +114) promoter region of human CXCL11 (IP9).

The promoter can also be derived from the -289 to +217 promoter region of human IGFBP4 (Insulin growth factor binding protein 4). The promoter can also be derived from the full length (-836 to +217) promoter region of human IGFBP4.

20   The promoter response element generally will be present in multiple copies, e.g., as tandem repeats. For example, in one reporter construct, coding sequence for luciferase is under control of an upstream 6X tandem repeat of NF-κB response element. In another example, an ISRE-luciferase reporter construct useful in the invention is available from Stratagene (catalog no. 219092) and includes a 5x ISRE tandem repeat joined to a TATA box

25   upstream of a luciferase reporter gene.

The reporter construct coding sequence is preferably any nucleotide sequence that codes for a protein capable of detection or quantification. The protein can be an enzyme (e.g., luciferase, alkaline phosphatase, β-galactosidase, chloramphenicol acetyltransferase (CAT), secreted alkaline phosphatase, etc.), a bioluminescence marker (e.g., green fluorescent protein

30   (GFP, U.S. Pat. No. 5,491,084), etc.), blue fluorescent protein (BFP, e.g., U.S. Pat. No. 6,486,382), etc.), a surface-expressed molecule (e.g., CD25, CD80, CD86), a secreted molecule (e.g., IL-1, IL-6, IL-8, IL-12 p40, TNF-α), a hapten or antigen, and other detectable protein products known to those of skill in the art. For assays relying on enzyme activity

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readout, substrate can be supplied as part of the assay, and detection can involve measurement of chemiluminescence, fluorescence, color development, incorporation of radioactive label, drug resistance, or other marker of enzyme activity. For assays relying on surface expression of a molecule, detection can be accomplished using flow cytometry (FACS) analysis or

5   functional assays. Secreted molecules can be assayed using enzyme-linked immunosorbent assay (ELISA) or bioassays. Many of these and other suitable readout systems are well known in the art and are commercially available. Preferably, the coding sequence encodes a protein having a level or an activity that is quantifiable, preferably with a wide linear range.

The expression construct coding sequence is preferably a TLR coding sequence
10   derived from the sequences listed herein. Preferably, the expression construct promoter is a constitutive promoter, although in some embodiments it may be inducible. Those of ordinary skill in the art are familiar with such promoters.

As used herein, a coding sequence and the regulatory sequences (such as promoters) are said to be operably linked when they are covalently linked in such a way as to place the
15   expression or transcription and/or translation of the coding sequence under the influence or control of the regulatory sequence. Two DNA sequences are said to be operably linked if induction of a promoter in the 5' regulatory sequence results in the transcription of the coding sequence and if the nature of the linkage between the two DNA sequences does not (1) result in the introduction of a frame-shift mutation, (2) interfere with the ability of the promoter
20   region to direct the transcription of the coding sequence, or (3) interfere with the ability of the corresponding RNA transcript to be translated into a protein. Thus, a regulatory sequence would be operably linked to a coding sequence if the gene expression sequence were capable of effecting transcription of that coding sequence such that the resulting transcript is translated into the desired protein or polypeptide.

25   Methods for nucleic acid introduction into cells are known in the art.

The nucleic acid may be delivered to the cells alone or in association with a vector. In its broadest sense, a vector is any vehicle capable of facilitating the transfer of the nucleic acid to the cells so that the reporter can be expressed. The vector generally transports the nucleic acid to the cells with reduced degradation relative to the extent of degradation that would
30   result in the absence of the vector. In general, the vectors useful in the invention include, but are not limited to, plasmids, phagemids, viruses, other vehicles derived from viral or bacterial sources that have been manipulated by the insertion or incorporation of the antigen nucleic acid sequences. Viral vectors are a preferred type of vector and include, but are not limited

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to, nucleic acid sequences from the following viruses: retrovirus, such as Moloney murine leukemia virus, Harvey murine sarcoma virus, murine mammary tumor virus, and Rous sarcoma virus; adenovirus, adeno-associated virus; SV40-type viruses; polyoma viruses; Epstein-Barr viruses; papilloma viruses; herpes virus; vaccinia virus; polio virus; and RNA
5   virus such as a retrovirus. One can readily employ other vectors not named but known in the art.

Preferred viral vectors are based on non-cytopathic eukaryotic viruses in which non-essential genes have been replaced with the gene of interest. Non-cytopathic viruses include retroviruses, the life cycle of which involves reverse transcription of genomic viral
10   RNA into DNA with subsequent proviral integration into host cellular DNA. Retroviruses have been approved for human gene therapy trials. Most useful are those retroviruses that are replication-deficient (i.e., capable of directing synthesis of the desired proteins, but incapable of manufacturing an infectious particle). Such genetically altered retroviral expression vectors have general utility for the high-efficiency transduction of genes *in vivo*. Standard
15   protocols for producing replication-deficient retroviruses (including the steps of incorporation of exogenous genetic material into a plasmid, transfection of a packaging cell lined with plasmid, production of recombinant retroviruses by the packaging cell line, collection of viral particles from tissue culture media, and infection of the target cells with viral particles) are provided in Kriegler, M., Gene Transfer and Expression, A Laboratory Manual W.H.
20   Freeman C.O., New York (1990) and Murray, E.J. Methods in Molecular Biology, vol. 7, Humana Press, Inc., Cliffton, New Jersey (1991).

A preferred virus for certain applications is the adeno-associated virus, a double-stranded DNA virus. The adeno-associated virus can be engineered to be replication -deficient and is capable of infecting a wide range of cell types and species. It further has
25   advantages such as, heat and lipid solvent stability; high transduction frequencies in cells of diverse lineages, including hemopoietic cells; and lack of superinfection inhibition thus allowing multiple series of transductions. Reportedly, wild-type adeno-associated virus manifest some preference for integration sites into human cellular DNA, thereby minimizing the possibility of insertional mutagenesis and variability of inserted gene expression
30   characteristic of retroviral infection. In addition, wild-type adeno-associated virus infections have been followed in tissue culture for greater than 100 passages in the absence of selective pressure, implying that the adeno-associated virus genomic integration is a relatively stable event. The adeno-associated virus can also function in an extrachromosomal fashion.

Recombinant adeno-associated viruses that lack the replicase protein apparently lack this integration sequence specificity.

Other vectors include plasmid vectors. Plasmid vectors have been extensively described in the art and are well-known to those of skill in the art. See e.g., Sambrook et al.,
5   Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, 1989. In the last few years, plasmid vectors have been found to be particularly advantageous for delivering genes to cells *in vivo* because of their inability to replicate within and integrate into a host genome. These plasmids, however, having a promoter compatible with the host cell, can express a peptide from a gene operatively encoded within the plasmid.
10 Some commonly used plasmids include pBR322, pUC18, pUC19, pRc/CMV, SV40, and pBlueScript. Other plasmids are well-known to those of ordinary skill in the art. Additionally, plasmids may be custom designed using restriction enzymes and ligation reactions to remove and add specific fragments of DNA.

In general, the vectors useful in the invention are divided into two classes: biological
15 vectors and chemical/physical vectors. Biological vectors and chemical/physical vectors are useful in the delivery and/or uptake of reporter constructs of the invention.

Most biological vectors are used for delivery of nucleic acids and thus would be most appropriate in the delivery of nucleic acids.

As used herein, a "chemical/physical vector" refers to a natural or synthetic molecule,
20 other than those derived from bacteriological or viral sources, capable of delivering the reference and test compound.

A preferred chemical/physical vector of the invention is a colloidal dispersion system. Colloidal dispersion systems include lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes. A preferred colloidal system of the invention is a
25 liposome. Liposomes are artificial membrane vessels which are useful as a delivery vector *in vivo* or *in vitro*. It has been shown that large unilamellar vessels (LUV), which range in size from 0.2 - 4.0 µm can encapsulate large macromolecules. RNA, DNA and intact virions can be encapsulated within the aqueous interior and be delivered to cells in a biologically active form (Fraley, et al., *Trends Biochem. Sci.*, (1981) 6:77).
30 Liposomes may be targeted to a particular tissue by coupling the liposome to a specific ligand such as a monoclonal antibody, sugar, glycolipid, or protein. Ligands which may be useful for targeting a liposome to an immune cell include, but are not limited to, intact or fragments of molecules which interact with immune cell specific receptors and molecules,

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such as antibodies, which interact with the cell surface markers of immune cells. Such

ligands may easily be identified by binding assays well known to those of skill in the art. In

still other embodiments, the liposome may be targeted to the cancer by coupling it to a one of

the immunotherapeutic antibodies discussed earlier. Additionally, the vector may be coupled

5   to a nuclear targeting peptide, which will direct the vector to the nucleus of the host cell.

Lipid formulations for transfection are commercially available from QIAGEN, for

example, as EFFECTENE™ (a non-liposomal lipid with a special DNA condensing enhancer)

and SUPERFECT™ (a novel acting dendrimeric technology).

Liposomes are commercially available from Gibco BRL, for example, as

10   LIPOFECTIN™ and LIPOFECTACE™, which are formed of cationic lipids such as N-[1-(2,

3 dioleyloxy)-propyl]-N, N, N-trimethylammonium chloride (DOTMA) and dimethyl

dioctadecylammonium bromide (DDAB). Methods for making liposomes are well known in

the art and have been described in many publications. Liposomes also have been reviewed by

Gregoriadis, G. in *Trends in Biotechnology*, (1985) 3:235-241. In some preferred

15   embodiments, the method of choice for delivering DNA (for transfection) to the cells is

electroporation, particularly where a stably transfected cell line is sought.

The present invention is further illustrated by the following Examples, which in no

way should be construed as further limiting.

20

<u>Examples</u>

Example 1. Biological Activity of Production Lot of CpG ODN (SEQ ID NO:1) Assayed

Using Cells Stably Transfected with hTLR9 Expression Vector

CpG ODN (SEQ ID NO:1) is currently in preclinical and clinical trials for a number of

25   clinical applications. SEQ ID NO:1 has been discovered to induce signaling through TLR9.

In order to assess different lots of clinical material, the methods of the invention are

employed, using a highly characterized lot of SEQ ID NO:1 as a reference.

In a TLR9 assay, the CpG-non-responsive human embryonal kidney cell line HEK293

(e.g., ATCC CRL-1573) was stably transfected with a hTLR9 expression construct and found

30   to express full-length human TLR9 constitutively. The cells also contained a genomic copy

of a reporter construct with a 6x NF-κB binding site and a luciferase gene reporter cassette.

Incubation of the cells with CpG ODN (SEQ ID NO:1) activates NF-κB driven expression of

luciferase, while incubation with medium alone (negative control) does not. The cells are

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then lysed and activity of the luciferase protein determined by its catalytic activity of luciferin oxidation which is measured in a luminometer. Results are expressed as fold induction above medium control.

Assay set-up includes a reference standard material which is highly pure and well
5 characterized. The reference material is used to create a standard curve within a defined range where the dose-response curve is linear (e.g., in the range of the EC50 value for SEQ ID NO:1, 70-100 nM). The test material is dissolved for testing and assayed at a defined concentration. Activity of the test material is calculated using the standard curve of the reference material. Quality of the tested material is deemed acceptable if activity of the test
10 material compared to activity of the reference material falls within predetermined limits.

Example 2. Biological Activity of Production Lot of CpG ODN (SEQ ID NO:1) Assayed Using RPMI 8226 Cells

The assay of Example 1 is performed using RPMI 8226 cells (ATCC CCL-155) in
15 place of the stably transfected HEK cells of Example 1. RPMI 8226 cells naturally express human TLR9. The cells are stably transfected with a 6x NF-κB-luciferase reporter construct. It is to be understood that the assay could also be carried out by measuring a native readout such as IL-10 secretion.

20 **Example 3. Expression Vectors for Human TLR3 (hTLR3) and Murine TLR3 (mTLR3)**

To create an expression vector for human TLR3, human TLR3 cDNA was amplified by the polymerase chain method (PCR) from a cDNA made from human 293 cells using the primers 5'-GAAACTCGAGCCACCATGAGACAGACTTTGCCTTGTATCTAC-3' (sense, SEQ ID NO:152) and 5'-GAAAGAATTCTTAATGTACAGAGTTTTTGGATCCAAG-3'
25 (antisense, SEQ ID NO:153). The primers introduce *XhoI* and *EcoRI* restriction endonuclease sites at their 5' ends for use in subsequent cloning into the expression vector. The resulting amplification product fragment was cloned into pGEM-T Easy vector (Promega), isolated, cut with *XhoI* and *EcoRI* restriction endonucleases, ligated into an *XhoI/EcoRI*-digested pcDNA3.1 expression vector (Invitrogen). The insert was fully sequenced and translated into
30 protein. The cDNA sequence corresponds to the published cDNA sequence for hTLR3, available as GenBank accession no. NM_003265 (SEQ ID NO:7). The open reading frame codes for a protein 904 amino acids long, having the sequence corresponding to GenBank accession no. NP_003256 (SEQ ID NO:8).

Corresponding nucleotide and amino acid sequences for murine TLR3 (mTLR3) are known. The nucleotide sequence of mTLR3 cDNA has been reported as GenBank accession no. AF355152 (SEQ ID NO:9), and the amino acid sequence of mTLR3 has been reported as GenBank accession no. AAK26117 (SEQ ID NO:10).

5

Example 4. Reconstitution of TLR3 Signaling in 293 Fibroblasts

Human TLR3 cDNA and murine TLR3 cDNA in pT-Adv vector (from Clontech) were individually cloned into the expression vector pcDNA3.1(-) from Invitrogen using the *EcoRI* site. The resulting expression vectors mentioned above were transfected into

10   CpG-DNA non-responsive human 293 fibroblast cells (ATCC, CRL-1573) using the calcium phosphate method. Utilizing a "gain of function" assay it was possible to reconstitute human TLR3 (hTLR3) and murine TLR3 (mTLR3) signaling in 293 fibroblast cells.

Since NF-κB activation is central to the IL-1/TLR signal transduction pathway (Medzhitov R et al. (1998) *Mol Cell* 2:253-8; Muzio M et al. (1998) *J Exp Med*

15   187:2097-101), in a first set of experiments human 293 fibroblast cells were transfected with hTLR3 alone or co-transfected with hTLR3 and an NF-κB-driven luciferase reporter construct.

Likewise, in a second set of experiments, 293 fibroblast cells were transfected with hTLR3 alone or co-transfected with hTLR3 and an IFN-α4-driven luciferase reporter

20   construct (described in Example 8 below).

In a third group of experiments, 293 fibroblast cells were transfected with hTLR3 alone or co-transfected with hTLR3 and a RANTES-driven luciferase reporter construct (described in Example 14 below).

25   **Example 5. Reconstitution of TLR7 Signaling**

Methods for cloning murine and human TLR7 have been described in pending U.S. Pat. Application No. 09/954,987 and corresponding published PCT application PCT/US01/29229 (WO 02/22809), both filed September 17, 2001, the contents of which are incorporated herein by reference. Human TLR7 cDNA and murine TLR7 cDNA in pT-Adv

30   vector (from Clontech) were individually cloned into the expression vector pcDNA3.1(-) from Invitrogen using the EcoRI site. Utilizing a "gain of function" assay it was possible to reconstitute human TLR7 (hTLR7) and murine TLR7 (mTLR7) signaling in CpG-DNA non-responsive human 293 fibroblasts (ATCC, CRL-1573). The expression vectors

mentioned above were transfected into 293 fibroblast cells using the calcium phosphate method.

Example 6. Reconstitution of TLR8 Signaling

5   Methods for cloning murine and human TLR8 have been described in pending U.S. Pat. Application No. 09/954,987 and corresponding published PCT application PCT/US01/29229 (WO 02/22809), both filed September 17, 2001, the contents of which are incorporated by reference. Human TLR8 cDNA and murine TLR8 cDNA in pT-Adv vector (from Clontech) were individually cloned into the expression vector pcDNA3.1(-) from

10   Invitrogen using the EcoRI site. Utilizing a "gain of function" assay it was possible to reconstitute human TLR8 (hTLR8) and murine TLR8 (mTLR8) signaling in CpG-DNA non-responsive human 293 fibroblasts (ATCC, CRL-1573). The expression vectors mentioned above were transfected into 293 fibroblast cells using the calcium phosphate method.

15

Example 7. Reconstitution of TLR9 Signaling in 293 Fibroblasts

          Methods for cloning murine and human TLR9 have been described in pending U.S. Pat. Application No. 09/954,987 and corresponding published PCT application PCT/US01/29229 (WO 02/22809), both filed September 17, 2001, the contents of which are

20   incorporated by reference. Human TLR9 cDNA and murine TLR9 cDNA in pT-Adv vector (from Clontech) were individually cloned into the expression vector pcDNA3.1(-) from Invitrogen using the EcoRI site. Utilizing a "gain of function" assay it was possible to reconstitute human TLR9 (hTLR9) and murine TLR9 (mTLR9) signaling in CpG-DNA non-responsive human 293 fibroblasts (ATCC, CRL-1573). The expression vectors

25   mentioned above were transfected into 293 fibroblast cells using the calcium phosphate method.

          To generate stable clones expressing human TLR9, murine TLR9, or either TLR9 with the NF-κB-luc reporter plasmid, 293 cells were transfected in 10 cm plates ($2x10^6$ cells/plate) with 16 µg of DNA and selected with 0.7 mg/ml G418 (PAA Laboratories GmbH, Cölbe,

30   Germany). Clones were tested for TLR9 expression by RT-PCR, for example as shown in Fig. 21. The clones were also screened for IL-8 production or NF-κB-luciferase activity after stimulation with ODN. Four different types of clones were generated.

293-hTLR9-luc: expressing human TLR9 and 6x NF-κB–luciferase reporter

293-mTLR9-luc: expressing murine TLR9 and 6x NF-κB–luciferase reporter

293-hTLR9:   expressing human TLR9

293-mTLR9:   expressing murine TLR9

5

     Human 293 fibroblast cells were transiently transfected with hTLR9 and a 6x

NF-κB-luciferase reporter plasmid (NF-κB-luc, kindly provided by Patrick Baeuerle, Munich,

Germany) (Fig. 18A) or with hTLR9 alone (Fig. 18B). After stimulus with CpG-ODN (2μM,

TCGTCGTTTTGTCGTTTTGTCGTT, SEQ ID NO:1), GpC-ODN (2μM,

10   TGCTGCTTTTGTGCTTTTGTGCTT, SEQ ID NO:154), LPS (100 ng/ml) or media, NF-κB

activation by luciferase readout (8h, Fig. 18A) or IL-8 production by ELISA (48h, Fig. 18B)

was monitored. Results are representative of three independent experiments. Fig. 18 shows

that cells expressing hTLR9 responded to CpG-DNA but not to LPS.

     Human 293 fibroblast cells were transiently transfected with mTLR9 and the

15   NF-κB-luc construct. Similar data was obtained for IL-8 production (not shown). Thus

expression of TLR9 (human or mouse) in 293 cells results in a gain of function for CpG DNA

stimulation similar to hTLR4 reconstitution of LPS responses.

     Figs. 19 and 20 demonstrate the responsiveness of a stable 293-mTLR9-luc and

293-hTLR9-luc clones after stimulation with CpG-ODN (2μM, SEQ ID NO:1), GpC-ODN

20   (2μM, SEQ ID NO:154), Me-CpG-ODN (2μM; TZGTZGTTTTGTZGTTTTGTZGTT, Z =

5-methylcytidine, SEQ ID NO:147), LPS (100 ng/ml) or media, as measured by monitoring

NF-κB activation. Similar results were obtained utilizing IL-8 production with the stable

clones. These results demonstrate that CpG-DNA non-responsive cell lines can be stably

genetically complemented with TLR9 to become responsive to CpG DNA in a motif-specific

25   manner.

Example 8. Method of Making IFN-α4 Reporter Vector

     A number of reporter vectors may be used in the practice of the invention. Some of

the reporter vectors are commercially available, e.g., the luciferase reporter vectors

30   pNF-κB-Luc (Stratagene) and pAP1-Luc (Stratagene). These two reporter vectors place the

luciferase gene under control of an upstream (5') promoter region derived from genomic DNA

for NF-κB or AP1, respectively. Other reporter vectors can be constructed following standard

methods using the desired promoter and a vector containing a suitable reporter, such as luciferase, β-galactosidase (β-gal), chloramphenicol acetyltransferase (CAT), and other reporters known by those skilled in the art. Following are some examples of reporter vectors constructed for use in the present invention.

5   IFN-α4 is an immediate-early type 1 IFN. Sequence-specific PCR products for the – 620 to +50 promoter region of IFN-α4 were derived from genomic DNA of human 293 cells and cloned into the *Sma*I site of the pGL3-Basic Vector (Promega). The resulting expression vector includes a luciferase gene under control of an upstream (5') –620 to +50 promoter region of IFN-α4. The sequence of the –620 to +50 promoter region of IFN-α4 is provided as

10   SEQ ID NO:121.

Example 9. Method of Making IFN-α1 Reporter Vector

         IFN-α1 is a late type 1 IFN. Sequence-specific PCR products for the –140 to +9 promoter region of IFN-α1 were derived from genomic DNA of human 293 cells and cloned

15   into *Sma*I site of the pGL3-Basic Vector (Promega). The resulting expression vector includes a luciferase gene under control of an upstream (5') –140 to +9 promoter region of IFN-α1. A sequence of the –140 to +9 promoter region of IFN-α1 is provided as SEQ ID NO:122.

Example 10. Method of Making IFN-β Reporter Vector

20   IFN-β is an immediate-early type 1 IFN. The –280 to +20 promoter region of IFN-β was derived from the pUCβ26 vector (Algarté M et al. (1999) *J Virol* 73:2694-702) by restriction at *Eco*RI and *Taq*I sites. The 300 bp restriction fragment was filled in by Klenow enzyme and cloned into *Nhe*I-digested and filled in pGL3-Basic Vector (Promega). The resulting expression vector includes a luciferase gene under control of an upstream (5') –280

25   to +20 promoter region of IFN-β. A sequence of the –280 to +20 promoter region of IFN-β is provided as SEQ ID NO:123.

Example 11. Method of Making Human IL-6 Reporter Vectors

         Reporter constructs are made using the -285 to +7 promoter region derived from

30   human IL-6 genomic DNA. (Takeshita et al. Eur. J. Immunol. 2000. 30: 108–116.) In one reporter construct the IL-6 promoter region is cloned as a *Kpn*I-*Xho*I insert into pGL3-Basic Vector (Promega). The resulting expression vector includes a luciferase gene under control of

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an upstream (5') -288 to +7 promoter region derived from human IL-6 genomic DNA. A
sequence of the –288 to +7 promoter region of human IL-6 is provided as SEQ ID NO:128.

The promoter can also be derived from the full-length promoter region of the IL-6
gene from –1174 to + 7 (GenBank Accession No M22111) as shown below as SEQ ID
5   NO:129.

Example 12. Method of Making Human IL-8 Reporter Vectors

Reporter constructs have been made using a -546 to +44 and a truncated -133 to +44
promoter region derived from human IL-8 genomic DNA. Mukaida N et al. (1989) *J*
10   *Immunol* 143:1366-71. In each reporter construct the IL-8 promoter region was cloned as a
*Kpn*I-*Xho*I insert into pGL3-Basic Vector (Promega). One of the resulting expression vectors
includes a luciferase gene under control of an upstream (5') -546 to +44 promoter region
derived from human IL-8 genomic DNA. Another of the resulting expression vectors
includes a luciferase gene under control of an upstream (5') -133 to +44 promoter region
15   derived from human IL-8 genomic DNA.

The promoter can also be the upstream (5') -734 to +44 or the upstream (5') -162 to
+44 promoter region derived from human IL-8 genomic DNA. Mukaida N et al. (1989) *J*
Immunol 143:1366-71. A sequence of the -734 to +44 promoter region derived from human
IL-8 is provided below as SEQ ID NO: 130.
20

Example 13. Method of Making Human IL-12 p40 Reporter Vectors

Reporter constructs have been made using truncated (-250 to +30, SEQ ID NO:127)
and full length (-751 to +30, SEQID NO:126) promoter regions derived from human IL-12
p40 genomic DNA. (Takeshita et al. Eur. J. Immunol. 2000. 30: 108–116.) In one reporter
25   construct the truncated IL-12 p40 promoter was cloned as a *Kpn*I-*Xho*I insert into pβgal-Basic
(Promega). The resulting expression vector includes a β gal gene under control of an
upstream (5') –250 to +30 promoter region of human IL-12 p40. In a second reporter
construct the full length IL-12 p40 promoter was cloned as a *Kpn*I-*Xho*I insert into
pβgal-Basic (Promega). The resulting expression vector includes a β gal gene under control
30   of an upstream (5') –751 to +30 promoter region of human IL-12 p40. In a third reporter
construct the truncated –250 to +30 promoter region of human IL-12 p40 was cloned into the
pGL3-Basic Vector (Promega). The resulting expression vector includes a luciferase gene
under control of an upstream (5') –250 to +30 promoter region of human IL-12 p40. In a

fourth reporter construct the full length IL-12 p40 promoter of human IL-12 p40 was cloned into the pGL3-Basic Vector (Promega). The resulting expression vector includes a luciferase gene under control of an upstream (5') –751 to +30 promoter region of human IL-12 p40. A sequence of the –751 to +30 promoter region of human IL-12 p40 is provided as SEQ ID NO: 126.

Example 14. Method of Making RANTES Reporter Vector

Transcription of the chemokine RANTES is believed to be regulated at least in part by IRF3 and by NF-κB. Lin R et al. (1999) *J Mol Cell Biol* 19(2):959-66; Genin P et al. (2000) *J Immunol* 164:5352-61. A 483 bp sequence-specific PCR product including the –397 to +5 promoter region of RANTES was derived from genomic DNA of human 293 cells, restricted with *Pst*I and cloned into pCAT-Basic Vector (Promega) using *Hind*III (filled in with Klenow) and *Pst*I sites (filled in). The –397 to +5 promoter region of RANTES was then isolated from the resulting RANTES/chloramphenicol acetyltransferase (CAT) reporter plasmid by restriction with *Bgl*II and *Sal*I, filled in with Klenow enzyme, and cloned into the Nhe*I* site (filled in with Klenow) of the pGL3-Basic Vector (Promega). The resulting expression vector includes a luciferase gene under control of an upstream (5') –397 to +5 promoter region of RANTES. Comparison of the insert sequence –397 to +5 of Genin P et al. (2000) *J Immunol* 164:5352-61 and GenBank accession no. AB023652 (SEQ ID NO:125) revealed two point deletions (at positions 105 and 273 of SEQ ID NO:125) which do not create new restriction sites. A sequence of the –397 to +5 promoter region of RANTES is provided as SEQ ID NO:125.

Example 15. RT-PCR Analysis of Cell Lines for TLR Expression

TLR expression was determined using total RNA of cells prepared by standard methods (QIAGEN). RNA was transcribed to cDNA using AMV Reverse Transcriptase (Roche). Quantitative PCR was performed with TLR-gene specific primer sets using a LightCycler Instrument (Roche). Controls for genomic DNA impurities were performed by a similar PCR method using RNA (but without reverse transciptase).

A variety of cell lines was screened for their expression of TLR3, 7, 8 and 9. These cell lines are A549 (human lung carcinoma), BeWo (human choriocarcinoma), HeLa (human cervix carcinoma), Hep-2 (human cervix carcinoma), KG-1 (human acute myeloid leukemia), MUTZ-3 (human acute myelomonocytic leukemia), Nalm-6 (human B cell precursor

leukemia), NK-92 (human Natural killer cell line), NK-92 MI (human Natural killer cell line, IL-2 independent), Raji (human Burkitt's lymphoma, B lymphocyte), RAMOS (Burkitt's lymphoma, B lymphocyte), RPMI 8226 (human multiple myeloma, B lymphocyte), THP-1 (human acute monocytic leukemia), U937 (human lymphoma) and Jurkat (human T cell

5   leukemia).

All B cell lines express, as determined by Real Time-PCR (RT-PCR), endogenous TLR9. In addition, all lines except NALM co-express TLR7. Nevertheless, none of the other cell lines appeared to express TLR7, whereas low TLR9 expression on the mRNA level was observed for KG-1 and THP-1. TLR3 appeared to be expressed in most of these cell lines,

10   with the highest mRNA levels for example in the NK cell lines (e.g., NK-92).

Raji cells contain high levels of TLR9 mRNA and low levels of TLR3 and TLR7 mRNA suggesting high expression of TLR9 protein and lower levels of TLR3 and TLR7 protein.

These results indicate that the cell lines expressing TLR9 can be used to screen

15   potential new TLR9 ligands (CpG ODN, etc.), cell lines expressing TLR7 to screen potential new TLR7 ligands (ORN (oligoribonucleotides), small molecules, etc.), and cell lines expressing both receptors may be used to screen for "hybrid" TLR7 and 9 agonists. In addition, cell lines lacking TLR8 expression (i.e., all cell lines tested) can be used to confirm the specificity of a TLR7 versus a TLR8 ligand (i.e., the latter should not be able to stimulate

20   TLR7-expressing cells). In contrast, cell lines expressing TLR3 (e.g., Raji cells) may be used to screen for potential new TLR3 ligands (dsRNA, etc.).

Example 16. Screening of Various Cell Lines for Responses to TLR Ligands

Except where otherwise indicated, the following general methods were used.

25   Cells were plated at 5 x 10^5/ml in 48 well plates in RPMI medium with 10% FBS. Stimulation was performed by addition of the oligonucleotides or other compounds diluted to the test concentrations in TE. Cells were incubated for 24 or 48h and the supernatants were taken to analyse for the presence of cytokines or chemokines.

The TLR ligands used are as follows:

30   TLR3: Poly I:C

TLR7, TLR8: R-848 .

TLR9:

T*C*C*A*G*G*A*C*T*T*C*T*C*T*C*A*G*G*T*T (SEQ ID NO: 2);

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T*C*G*T*C*G*T*T*T*T*G*T*C*G*T*T*T*T*G*T*C*G*T*T (SEQ ID NO: 1);

T*G*C*T*G*C*T*T*T*T*G*T*G*C*T*T*T*T*G*T*G*C*T*T (SEQ ID NO: 154);

T*C*G*T*C*G*T*T*T*T*C*G*G*C*G*C*G*C*G*C*C*G (SEQ ID NO: 158);

G*G*G_G_A_C_G_A_C_G_T_C_G_T_G_G*G*G*G*G*G (SEQ ID NO: 159);

5   T*G*C*T*G*C*T*T*T*T*C*G*G*C*G*G*C*C*G*C*C*G (SEQ ID NO: 160);

G*G*G_G_A_G_C_A_G_C_T_G_C_T_G_G*G*G*G*G*G (SEQ ID NO: 161).

* phosphorothioate linkage; _ phosphodiester linkage.

10   Increased expression of cell surface markers was determined using cells stimulated as
described above and then stained with different monoclonal antibody combinations specific
for the cell surface markers. Analysis of the cells was performed by flow cytometry.

Changes in reporter gene activity were determined using cells transfected with a
NF-κB reporter construct (Stratagene) and a β-galactosidase reporter control plasmid
(Invitrogen) using electroporation. For NF-κB analysis, a 5x NF-κB-Luciferase Vector
15   (Stratagene) was used. The amount of DNA transfected as well as cell concentration was
varied. Stimulation was performed 24h after transfection. Cells were stimulated with the
indicated amounts of ODN, R-848, LPS, TNF-α, or IL-1 β for the indicated incubation times.
Cell extracts were prepared by lysing the cells in 100 μl reporter lysis buffer (Promega) using
the freeze-thaw method. All data were normalized for β-galactosidase expression.
20   Stimulation indices were calculated in reference to luciferase activity of medium without
addition of ODN.

Stimulation of the Raji cell line with a TLR9 ligand (CpG ODN), a TLR3 ligand (poly
I:C) or a TLR7 ligand (R-848) results in the ligand-specific secretion of cytokines. Figs. 14
and 15 show IL-6 production of Raji cells upon stimulation with ODN, poly I:C or R-848.
25   Fig. 16 shows IFN-α2 production of Raji cells upon stimulation with ODN, poly I:C or R-848.
In all assays, cells were incubated with Na-Butyrate for 48h before stimulation with TLR
ligands. CpG stimulation of the RAMOS cell lines can result in the CpG-specific up-
regulation of cell surface markers such as CD80, as shown in Fig. 17.

30   **Example 17. Inhibition of a Positive Reference Compound Response with an Inhibitory
Test Compound**

Inhibition of CpG mediated chemokine production was determined using RPMI 8226
cells incubated with increasing amounts of SEQ ID NO:1 in the presence of an

immunoinhibitory ODN (SEQ ID NO: 151). IP-10 production was measured 24h later by ELISA (Fig. 9).

Equivalents

5   The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the invention. The present invention is not to be limited in scope by examples provided, since the examples are intended as a single illustration of one aspect of the invention and other functionally equivalent embodiments are within the scope of the invention. Various modifications of the invention in addition to those shown and described

10   herein will become apparent to those skilled in the art from the foregoing description and fall within the scope of the appended claims. The advantages and objects of the invention are not necessarily encompassed by each embodiment of the invention.

All references, patents and patent publications that are recited in this application are incorporated in their entirety herein by reference.

15

We claim:

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Claims

1.   A screening method for identifying agonists of Toll-like receptor (TLR) signaling activity, comprising

contacting an RPMI 8226 cell that expresses a TLR with a test compound and

5   measuring a test level of TLR signaling activity,

wherein a test level that is positive is indicative of a test compound that is a TLR agonist, and

wherein the TLR signaling activity is selected from the group consisting of CD71 expression, CD86 expression, HLA-DR expression, IL-8 expression, IL-8 production, IL-8

10   secretion, IL-10 expression, IL-10 production, IL-10 secretion, IP-10 expression, IP-10 production, IP-10 secretion, TNF-α expression, TNF-α production and TNF-α secretion.

2.   A screening method for identifying agonists of Toll-like receptor (TLR) signaling activity, comprising

15   contacting a cell that expresses a TLR with a test compound and measuring a test level of TLR signaling activity,

wherein a test level that is positive is indicative of an immunostimulatory compound, and

wherein the cell is a Raji cell, a RAMOS cell, a Nalm cell, a THP-1 cell, or a KG-

20   1 cell.

3.   The method of claim 1 or 2, wherein the test level is positive relative to a reference level determined by contacting the cell with a reference compound and measuring a reference TLR signaling activity.

25

4.   The method of claim 3, wherein the reference compound is a positive reference compound

5.   The method of claim 4, wherein the positive reference compound is

30   selected from the group consisting of an immunostimulatory nucleic acid and an imidazoquinoline compound.

6.   The method of claim 3, wherein the reference compound is a negative reference compound.

7.   The method of claim 6, wherein the negative reference compound is medium alone.

8.   The method of claim 5, wherein the immunostimulatory nucleic acid is selected from the group consisting of a CpG nucleic acid, a T-rich nucleic acid, a poly-T nucleic acid and a poly-G nucleic acid.

9.   The method of claim 5, wherein the imidazoquinoline compound is selected from the group consisting of R-848 and R-847.

10.   The method of claim 1 or 2, wherein the test compound is a nucleic acid.

11.   The method of claim 10, wherein the nucleic acid does not comprise a motif selected from the group consisting of a CpG motif, a poly-T motif, a T-rich motif and a poly-G motif.

12.   The method of claim 10, wherein the nucleic acid comprises a phosphorothioate backbone linkage.

13.   The method of claim 10, wherein the nucleic acid is a DNA, an RNA or a DNA-RNA hybrid.

14.   The method of claim 1 or 2, wherein the test compound is a non-nucleic acid small molecule.

15.   The method of claim 1 or 2, wherein the test compound comprises an amino acid, a carbohydrate, a lipid, or a hormone.

16.   The method of claim 15, wherein the carbohydrate is a polysaccharide.

17.   The method of claim 1 or 2, wherein the test compound is derived from
a molecular library.

5   18.   The method of claim 1, wherein the cell is transfected with a nucleic
acid.

19.   The method of claim 18, wherein the nucleic acid encodes a TLR or a
reporter construct.

10

20 .   The method of claim 2, wherein the cell is transfected with a nucleic
acid.

21.   The method of claim 20, wherein the nucleic acid encodes a TLR or a
15   reporter construct.

22.   The method of claim 19 or 21, wherein the TLR is selected from the
group consisting of TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9 and
TLR10.

20

23.   The method of claim 22, wherein the TLR is a human TLR.

24.   The method of claim 19 or 21, wherein the reporter construct is
selected from the group consisting of a luciferase reporter construct, a β-galactosidase reporter
25   construct, a chloramphenicol acetyltransferase reporter construct, a green fluorescent protein
reporter construct, and a secreted alkaline phosphatase construct.

25.   The method of claim 19 or 21, wherein the reporter construct comprises
a TLR responsive promoter.

30

26.   The method of claim 25, wherein the TLR responsive promoter
comprises a transcription factor binding site selected from the group consisting of a NF-κB
binding site, an AP-1 binding site, a CRE, a SRE, an ISRE, a GAS, an ATF2 binding site, an

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IRF3 binding site, an IRF7 binding site, an NFAT binding site, a p53 binding site, an SRF
binding site, and a TARE.

27.   The method of claim 25, wherein the TLR responsive promoter is a
promoter region selected from the group consisting of an IL-1 promoter region, an IL-6
promoter region, an IL-8 promoter region, an IL-10 promoter region, an IL-12 p40 promoter
region, an IFN-α1 promoter region, an IFN-α4 promoter region, an IFN-β promoter region, an
IFN-γ promoter region, a TNF-α promoter region, a TNF-β promoter region, an IP-9 promoter
region, an IP-10 promoter region, a RANTES promoter region, an ITAC promoter region, a
MCP-1 promoter region, an IGFBP4 promoter region, a CD54 promoter region, a CD69
promoter region, a CD71 promoter region, a CD80 promoter region, a CD86 promoter region,
a HLA-DR promoter region, and a HLA class I promoter region.

28.   The method of claim 18 or 20, wherein the cell is stably transfected.

29.   The method of claim 1 or 2, wherein the TLR signaling activity is
measured by cytokine secretion or chemokine secretion.

30.   The method of claim 1, wherein the TLR signaling activity is selected
from the group consisting of IL-8 secretion, IL-10 secretion, IP-10 secretion and TNF-α
secretion.

31.   The method of claim 2, wherein the TLR signaling activity is selected
from the group consisting of IL-6 expression, IL-6 production, IL-6 secretion, IL-8
expression, IL-8 production, IL-8 secretion, IL-10 expression, IL-10 production, IL-10
secretion, IP-10 expression, IP-10 production, IP-10 secretion, IL-12 expression, IL-12
production, IL-12 secretion, TNF-α expression, TNF-α production and TNF-α secretion.

32.   The method of claim 2, wherein the TLR signaling activity is measured
by phosphorylation.

33.   The method of claim 32, wherein phosphorylation is total cellular
phosphorylation.

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34.   The method of claim 32, wherein phosphorylation is phosphorylation of a factor selected from the group consisting of IRAK, ERK, MyD88, TRAF6, p38, NFkB subunits, c-Jun and c-Fos.

5

35.   The method of claim 1 or 2, wherein the TLR signaling activity is measured by gene expression.

36.   The method of claim 1, wherein the TLR signaling activity is measured

10   by gene expression selected from the group consisting of CD71 expression, CD86 expression, HLA-DR expression, IL-8 expression, IL-10 expression, IP-10 expression, and TNF-α expression.

37.   The method of claim 35, wherein TLR signaling activity is measured

15   by microarray techniques.

38.   The method of claim 2, wherein the TLR signaling activity is measured by cell proliferation.

20   39.   The method of claim 1 or 2, wherein TLR signaling activity is measured by cell surface marker expression.

40.   The method of claim 1, wherein TLR signaling activity is measured by cell surface expression of CD71, CD86 or HLA-DR.

25

41.   The method of claim 2, wherein TLR signaling activity is measured by CD71 cell surface expression, CD86 cell surface expression, HLA-DR cell surface expression, CD80 cell surface expression, HLA class I cell surface expression, CD54 cell surface expression and CD69 cell surface expression.

30

42.   The method of claim 2, wherein TLR signaling activity is measured by antibody secretion.

43. The method of claim 42, wherein the antibody secretion is IgM secretion.

44.   A composition comprising

an RPMI 8226 cell stably transfected with a nucleic acid encoding a TLR

5   polypeptide, or a fragment thereof.

45.   The composition of claim 44, further comprising a reporter construct

comprising a promoter and a reporter sequence wherein the promoter is a TLR responsive

promoter.

10

46.   The composition of claim 45, wherein the TLR responsive promoter

comprises a nucleic acid sequence selected from the group consisting of an NF-κB binding

site, an AP-1 binding site, a CRE, a SRE, an ISRE, a GAS, an ATF2 binding site, an IRF3

binding site, an IRF7 binding site, an NFAT binding site, a p53 binding site, an SRF binding

15   site, and a TARE.

47.   The composition of claim 45, wherein the reporter sequence is selected

from the group consisting of a luciferase sequence, a β-galactosidase sequence, a green

fluorescent protein sequence, a secreted alkaline phosphatase sequence and a chloramphenicol

20   transferase sequence.

48.   The composition of claim 44, wherein the TLR polypeptide or fragment

thereof is a human TLR polypeptide or fragment thereof.

25   49.   The composition of claim 44, wherein the TLR polypeptide or fragment

thereof is selected from the group consisting of TLR1, TLR2, TLR3, TLR4, TLR5, TLR6,

TLR7, TLR8, TLR9 and TLR10.

50.   The composition of claim 44, wherein the TLR polypeptide or fragment

30   thereof is a human TLR polypeptide.

51.   A screening method for identifying agonists of Toll-like receptor (TLR)

signaling activity, comprising

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contacting an cell that ectopically expresses a TLR with a test compound and measuring a test level of TLR signaling activity,

wherein a test level that is positive is indicative of a test compound that is a TLR agonist, and

5   wherein the cell that ectopically expresses a TLR is selected from the group consisting of RPMI 8226, RAMOS, Raji, Nalm, THP-1, KG-1 and 293 HEK.

52.   The method of claim 51, wherein the test level is positive relative to a reference level determined by contacting the cell with a reference compound and measuring a

10   reference TLR signaling activity.

53.   The method of claim 52, wherein the reference compound is a positive reference compound.

15   54.   The method of claim 53, wherein the positive reference compound is selected from the group consisting of an immunostimulatory nucleic acid and an imidazoquinoline compound.

55.   The method of claim 54, wherein the immunostimulatory nucleic acid

20   is selected from the group consisting of a CpG nucleic acid, a T-rich nucleic acid, a poly-T nucleic acid and a poly-G nucleic acid.

56.   The method of claim 54, wherein the imidazoquinoline compound is selected from the group consisting of R-848 and R-847.

25

57.   The method of claim 52, wherein the reference compound is negative reference compound.

58.   The method of claim 57, wherein the negative reference compound is

30   medium alone.

59.   The method of claim 51, wherein the test compound is a nucleic acid.

60.   The method of claim 59, wherein the nucleic acid does not comprise a motif selected from the group consisting of a CpG motif, a poly-T motif, a T-rich motif and a poly-G motif.

61.   The method of claim 59, wherein the nucleic acid comprises a phosphorothioate backbone linkage.

62.   The method of claim 59, wherein the nucleic acid is a DNA, an RNA, or a DNA-RNA hybrid.

63.   The method of claim 51, wherein the test compound is a non-nucleic acid small molecule.

64.   The method of claim 51, wherein the test compound comprises an amino acid, a carbohydrate, a lipid, or a hormone.

65.   The method of claim 64, wherein the carbohydrate is a polysaccharide.

66.   The method of claim 51, wherein the test compound is derived from a molecular library.

67.   The method of claim 51, wherein the TLR signaling activity is selected from the group consisting of CD71 expression, CD86 expression, HLA-DR expression, IL-6 expression, IL-6 production, IL-6 secretion, IL-8 expression, IL-8 production, IL-8 secretion, IL-10 expression, IL-10 production, IL-10 secretion, IL-12 expression, IL-12 production, IL-12 secretion, IP-10 expression, IP-10 production, IP-10 secretion, TNF-α expression, TNF-α production and TNF-α secretion.

68.   The method of claim 51, wherein the TLR is selected from the group consisting of TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9 and TLR10.

69.   The method of claim 51, wherein the TLR is a human TLR.

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70.   The method of claim 51, wherein the cell is transfected with a reporter construct.

71.   The method of claim 70, wherein the reporter construct is selected from
5   the group consisting of a luciferase reporter construct, a β-galactosidase reporter construct, a
chloramphenicol acetyltransferase reporter construct, a green fluorescent protein reporter
construct, and a secreted alkaline phosphatase construct.

72.   The method of claim 71, wherein the TLR signaling activity is
10 measured by luciferase expression, β-galactosidase expression, chloramphenicol expression,
acetyltransferase expression, green fluorescent protein expression, alkaline phosphatase
expression and alkaline phosphatase secretion.

73.   The method of claim 71, wherein the reporter construct comprises a
15 TLR responsive promoter.

74.   The method of claim 25 or 73, wherein the TLR responsive promoter is
a TLR1 responsive promoter, a TLR2 responsive promoter, a TLR3 responsive promoter, a
TLR4 responsive promoter, a TLR5 responsive promoter, a TLR6 responsive promoter, a
20 TLR7 responsive promoter, a TLR8 responsive promoter, a TLR9 responsive promoter and a
TLR10 responsive promoter.

75.   The method of claim 73, wherein the TLR responsive promoter
comprises a transcription factor binding site selected from the group consisting of an NF-κB
25 binding site, an AP-1 binding site, a CRE, a SRE, an ISRE, a GAS, an ATF2 binding site, an
IRF3 binding site, an IRF7 binding site, an NFAT binding site, a p53 binding site, an SRF
binding site, and a TARE.

76.   The method of claim 73, wherein the TLR responsive promoter is a
30 promoter region selected from the group consisting of an IL-1 promoter region, an IL-6
promoter region, an IL-8 promoter region, an IL-10 promoter region, an IL-12 p40 promoter
region, an IFN-α1 promoter region, an IFN-α4 promoter region, an IFN-β promoter region, an
IFN-γ promoter region, a TNF-α promoter region, a TNF-β promoter region, an IP-9 promoter

region, an IP-10 promoter region, a RANTES promoter region, an ITAC promoter region, a MCP-1 promoter region, an IGFBP4 promoter region, a CD54 promoter region, a CD69 promoter region, a CD71 promoter region, a CD80 promoter region, a CD86 promoter region, a HLA-DR promoter region, and a HLA class I promoter region.

77.   The method of claim 51, wherein the cell is stably transfected with a TLR nucleic acid.

78.   The method of claim 70, wherein the cell is stably transfected with the reporter construct.

79.   The method of claim 51, wherein the TLR signaling activity is measured by cytokine secretion or chemokine secretion.

80.   The method of claim 79, wherein the cytokine secretion or chemokine secretion is selected from the group consisting of IL-8 secretion, TNF-α secretion, IL-10 secretion and IP-10 secretion.

81.   The method of claim 79, wherein the cytokine secretion or chemokine secretion is selected from the group consisting of IL-6 secretion and IL-12 secretion.

82.   The method of claim 51, wherein the TLR signaling activity is measured by phosphorylation.

83.   The method of claim 82, wherein phosphorylation is total cellular phosphorylation.

84.   The method of claim 82, wherein phosphorylation is phosphorylation of a factor selected from the group consisting of IRAK, ERK, MyD88, TRAF6, p38, NF-κB subunits, c-Jun and c-Fos.

85.   The method of claim 51, wherein the TLR signaling activity is measured by gene expression.

86.   The method of claim 85, wherein the gene expression is selected from the group consisting of IL-8 expression, IL-10 expression, IP-10 expression, CD71 expression, CD86 expression and HLA-DR expression.

87.   The method of claim 85, wherein the gene expression is selected from the group consisting of IL-6 expression, IL-12 expression and TNF-α expression.

88.   The method of claim 51, wherein the TLR signaling activity is measured by microarray techniques.

89.   The method of claim 51, wherein the TLR signaling activity is measured by cell proliferation.

90.   The method of claim 51, wherein the TLR signaling activity is measured by cell surface marker expression.

91.   The method of claim 90, wherein the cell surface marker expression is selected from the group consisting of CD71 cell surface expression, CD86 cell surface expression and HLA-DR cell surface expression.

92.   The method of claim 90, wherein the cell surface marker expression is selected from the group consisting of CD80 cell surface expression, HLA class I cell surface expression, CD54 cell surface expression and CD69 cell surface expression.

93.   The method of claim 51, wherein the TLR signaling activity is measured by antibody secretion.

94.   The method of claim 93, wherein the antibody secretion is IgM secretion.

95.   A screening method for identifying antagonists of Toll-like receptor (TLR) signaling activity, comprising

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contacting a cell with a positive reference compound and measuring a reference
level of TLR signaling activity,

contacting the cell with the positive reference compound and a test compound,
and measuring a test level of TLR signaling activity,

5   wherein a test level that is less than a reference level is indicative of test compound
that is a TLR antagonist, and

wherein the cell is selected from the group consisting of a RPMI 8226 cell, a
RAMOS cell, a Raji cell, a THP-1 cell, a Nalm cell and a KG-1 cell.

10   96.   The method of claim 95, wherein the positive reference compound is
selected from the group consisting of an immunostimulatory nucleic acid and an
immunostimulatory imidazoquinoline compound.

97.   The method of claim 96, wherein the immunostimulatory nucleic acid
15 is selected from the group consisting of a CpG nucleic acid, a T-rich nucleic acid, a poly-T
nucleic acid and a poly-G nucleic acid.

98.   The method of claim 96, wherein the imidazoquinoline compound is
selected from the group consisting of R-848 and R-847.

20

99.   The method of claim 95, wherein the test compound is a nucleic acid.

100.   The method of claim 99, wherein the nucleic acid does not comprise a
motif selected from the group consisting of a CpG motif, a poly-T motif, a T-rich motif and a
25 poly-G motif.

101.   The method of claim 99, wherein the nucleic acid comprises a
phosphorothioate backbone linkage.

30   102.   The method of claim 99, wherein the nucleic acid is a DNA, an RNA or
a DNA-RNA hybrid.

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103.   The method of claim 95, wherein the test compound is a non-nucleic acid small molecule.

104.   The method of claim 95, wherein the test compound comprises an amino acid, a carbohydrate, a lipid, or a hormone.

105.   The method of claim 104, wherein the carbohydrate is a polysaccharide.

106.   The method of claim 95, wherein the test compound is derived from a molecular library.

107.   The method of claim 95, wherein the experimental cell is transfected with a nucleic acid.

108.   The method of claim 107, wherein the nucleic acid encodes a TLR or a reporter construct.

109.   The method of claim 108, wherein the TLR is selected from the group consisting of TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9 and TLR10.

110.   The method of claim 108, wherein the TLR is a human TLR.

111.   The method of claim 108, wherein the reporter construct is selected from the group consisting of a luciferase reporter construct, a β-galactosidase reporter construct, a chloramphenicol acetyltransferase reporter construct, a green fluorescent protein reporter construct, and a secreted alkaline phosphatase construct.

112.   The method of claim 111, wherein the TLR signaling activity is selected from the group consisting of luciferase expression, β-galactosidase expression, chloramphenicol acetyltransferase expression, green fluorescent protein expression, alkaline phosphatase expression and alkaline phosphatase secretion.

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113.   The method of claim 108, wherein the reporter construct comprises a
TLR responsive promoter.

114.   The method of claim 113, wherein the TLR responsive promoter
5   comprises a transcription factor binding site selected from the group consisting of an NF-κB
binding site, an AP-1 binding site, a CRE, a SRE, an ISRE, a GAS, an ATF2 binding site, an
IRF3 binding site, an IRF7 binding site, an NFAT binding site, a p53 binding site, an SRF
binding site, and a TARE.

10   115.   The method of claim 113, wherein the TLR responsive promoter is a
promoter region selected from the group consisting of an IL-1 promoter region, an IL-6
promoter region, an IL-8 promoter region, an IL-10 promoter region, an IL-12 p40 promoter
region, an IFN-α1 promoter region, an IFN-α4 promoter region, an IFN-β promoter region, an
IFN-γ promoter region, a TNF-α promoter region, a TNF-β promoter region, an IP-9 promoter
15   region, an IP-10 promoter region, a RANTES promoter region, an ITAC promoter region, a
MCP-1 promoter region, an IGFBP4 promoter region, a CD54 promoter region, a CD69
promoter region, a CD71 promoter region, a CD80 promoter region, a CD86 promoter region,
a HLA-DR promoter region, and a HLA class I promoter region.

20   116.   The method of claim 113, wherein the TLR responsive promoter is
selected from the group consisting of a TLR1 responsive promoter, TLR2 responsive
promoter, a TLR3 responsive promoter, a TLR4 responsive promoter, a TLR5 responsive
promoter, a TLR6 responsive promoter, a TLR7 responsive promoter, a TLR8 responsive
promoter, a TLR9 responsive promoter and a TLR10 responsive promoter.
25

117.   The method of claim 107, wherein the cell is stably transfected with the
nucleic acid.

118.   The method of claim 95, wherein the TLR signaling activity is
30   measured by cytokine secretion or chemokine secretion.

119. The method of claim 118, wherein the cytokine secretion or chemokine secretion is selected from the group consisting of IL-6 secretion, IL-12 secretion and TNF-α secretion.

5   120. The method of claim 118, wherein the cytokine secretion or chemokine secretion is selected from the group consisting of IL-8 secretion, IL-10 secretion and IP-10 secretion.

121. The method of claim 95, wherein the TLR signaling activity is
10   measured by phosphorylation.

122. The method of claim 121, wherein phosphorylation is total cellular phosphorylation.

15   123. The method of claim 122, wherein phosphorylation is phosphorylation of a factor selected from the group consisting of IRAK, ERK, MyD88, TRAF6, p38, NF-κB subunits, c-Jun and c-Fos.

124. The method of claim 95, wherein the TLR signaling activity is
20   measured by gene expression.

125. The method of claim 124, wherein the gene expression is selected from the group consisting of CD71 expression, CD86 expression, HLA-DR expression, IL-8 expression, IL-10 expression and IP-10 expression.

25

126. The method of claim 124, wherein the gene expression is selected from the group consisting of IL-6 expression, IL-12 expression and TNF-α expression.

127. The method of claim 95, wherein the TLR signaling activity is
30   measured by microarray techniques.

128. The method of claim 95, wherein the TLR signaling activity is measured by cell proliferation.

129.   The method of claim 95, wherein the TLR signaling activity is measured by cell surface marker expression.

5   130.   The method of claim 129, wherein the cell surface marker expression is selected from the group consisting of CD71 cell surface expression, CD86 cell surface expression and HLA-DR MHC class II cell surface expression.

131.   The method of claim 129, wherein the cell surface marker expression is 10   selected from the group consisting of CD80 cell surface expression, HLA class I cell surface expression, CD54 cell surface expression and CD69 cell surface expression.

132.   The method of claim 95, wherein the TLR signaling activity is measured by antibody secretion.

15

133.   The method of claim 132, wherein the antibody secretion is IgM secretion.

134.   The method of claim 95, wherein the cell is contacted to the positive 20   reference compound and the test compound simultaneously.

135.   The method of claim 95, wherein the cell is contacted to the positive reference compound prior to contact with the test compound.

25   136.   The method of claim 95, wherein the cell is contacted to the test compound prior to contact with the positive reference compound.

137.   A method for quality assessment of a test composition containing a known Toll like receptor (TLR) ligand, comprising:

30   measuring a reference activity of a reference composition comprising a known TLR ligand, wherein the known TLR ligand is a nucleic acid molecule;

measuring a test activity of a test composition comprising the known TLR ligand; and

comparing the test activity to the reference activity.

138.   The method of claim 137, further comprising selecting the test
composition if the test activity falls within a predetermined range of variance about the
reference activity.

5

139.   The method of claim 1, wherein the reference composition is a first
production lot of a pharmaceutical composition comprising the known TLR ligand, and
wherein the test composition is a second production lot of a pharmaceutical composition
comprising the known TLR ligand.

10

140.   The method of claim 137, wherein the reference composition is a first
in-process lot of a composition comprising the known TLR ligand, and wherein the test
composition is a second in-process lot of a composition comprising the known TLR ligand.

15   141.   The method of claim 137, wherein the measuring the reference activity
comprises contacting the reference composition with an isolated cell expressing a TLR
responsive to the known TLR ligand, and wherein the measuring the test activity comprises
contacting the test composition with the isolated cell expressing a TLR responsive to the
known TLR ligand.

20

142.   The method of claim 141, wherein the isolated cell expressing the TLR
responsive to the known TLR ligand comprises an expression vector for the TLR responsive
to the known TLR ligand.

25   143.   The method of claim 141, wherein the isolated cell expressing the TLR
responsive to the known TLR ligand naturally expresses the TLR responsive to the known
TLR ligand.

144.   The method of claim 141, wherein the isolated cell expressing the TLR
30   responsive to the known TLR ligand is RPMI 8226.

145.   The method of claim 137, wherein the measuring the reference activity and the measuring the test activity each comprise measuring signaling activity mediated by a TLR responsive to the known TLR ligand.

5   146.   The method of claim 145, wherein the signaling activity is activity of a reporter construct under control of NF-κB response element.

147.   The method of claim 145, wherein the signaling activity is activity of a reporter construct under control of interferon-stimulated response element (ISRE).

10

148.   The method of claim 145, wherein the signaling activity is activity of a reporter gene under control of an IFN-α promoter.

149.   The method of claim 145, wherein the signaling activity is activity of a
15   reporter gene under control of an IFN-β promoter.

150.   The method of claim 145, wherein the signaling activity is activity of a reporter gene under control of an IL-6 promoter.

20   151.   The method of claim 145, wherein the signaling activity is activity of a reporter gene under control of an IL-8 promoter.

152.   The method of claim 145, wherein the signaling activity is activity of a reporter gene under control of an IL-12 p40 promoter.

25

153.   The method of claim 145, wherein the signaling activity is activity of a reporter gene under control of a RANTES promoter.

154.   The method of claim 137, wherein the known TLR ligand is a TLR9
30   ligand.

155.   The method of claim 137, wherein the known TLR ligand is a TLR3 ligand.

156.   The method of claim 137, wherein the known TLR ligand is a TLR7 ligand.

5   157.   The method of claim 137, wherein the known TLR ligand is a TLR8 ligand.

158.   The method of claim 137, wherein the known TLR ligand is an immunostimulatory nucleic acid.

10

159.   The method of claim 137, wherein the known TLR ligand is a CpG nucleic acid.

160.   The method of claim 137, wherein the known TLR ligand is an
15   immunoinhibitory nucleic acid.

161.   A method for quality assessment of a test lot of a pharmaceutical product containing a known TLR9 ligand, comprising:
        measuring a reference activity of a reference lot of a pharmaceutical product
20   comprising a known TLR9 ligand, wherein the known TLR9 ligand is a nucleic acid molecule;
        measuring a test activity of a test lot of a pharmaceutical product comprising the known TLR9 ligand;
        comparing the test activity to the reference activity; and
25   rejecting the test lot if the test activity falls outside of a predetermined range of variance about the reference activity.

162.   The method of claim 161, wherein the known TLR9 ligand is an oligonucleotide comprising a base sequence TCGTCGTTTTGTCGTTTTGTCGTT (SEQ ID
30   NO:1).

163.   The method of claim 161, wherein the known TLR9 ligand is an oligonucleotide comprising a base sequence 5'-TCGTCGTTTTGACGTTTTGTCGTT-3' (SEQ ID NO:139).

5   164.   The method of claim 161, wherein the known TLR9 ligand is an oligonucleotide comprising a base sequence 5'-TCGTCGTTTTGTCGTTTTTTTCGA-3' (SEQ ID NO:140).

165.   The method of claim 161, wherein the known TLR9 ligand is an
10 oligonucleotide comprising a base sequence 5'-TCGTCGTTTCGTCGTTTCGTCGTT-3' (SEQ ID NO:141).

166.   The method of claim 161, wherein the known TLR9 ligand is an oligonucleotide comprising a base sequence 5'-TCGTCGTTTCGTCGTTTTGTCGTT-3'
15 (SEQ ID NO:142).

167.   The method of claim 161, wherein the known TLR9 ligand is an oligonucleotide comprising a base sequence 5'-TCGTCGTTTTTCGGTCGTTTT-3' (SEQ ID NO:143).

20

168.   The method of claim 161, wherein the known TLR9 ligand is an oligonucleotide comprising a base sequence 5'-TCGTCGTTTTTCGTGCGTTTTT-3' (SEQ ID NO:144).

25   169.   The method of claim 161, wherein the known TLR9 ligand is an oligonucleotide comprising a base sequence 5'-TCGTCGTTTTCGGCGGCCGCCG-3' (SEQ ID NO:145).

170.   The method of claim 161, wherein the known TLR9 ligand is an
30 oligonucleotide comprising a base sequence 5'-TCGTC_GTTTTAC_GGCGCC_GTGCCG-3' (SEQ ID NO:146), wherein every internucleoside linkage is phosphorothioate except for those indicated by "_", which are phosphodiester.

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171.   A screening method for identifying agonists of Toll-like receptor (TLR) signaling activity, comprising

contacting a cell that expresses a TLR with a test compound and measuring a test level of TLR signaling activity,

5   wherein a test level that is positive is indicative of a test compound that is a TLR agonist, and

wherein the cell is a Raji cell, a RAMOS cell, a Nalm cell, a THP-1 cell, or a KG-1 cell, and the TLR is TLR9.

10   172.   A screening method for identifying agonists of Toll-like receptor (TLR) signaling activity, comprising

contacting a cell that expresses a TLR with a test compound and measuring a test level of TLR signaling activity,

wherein a test level that is positive is indicative of a test compound that is a TLR

15   agonist, and

wherein the cell is a Raji cell or a RAMOS cell, and the TLR is TLR7.

173.   A screening method for identifying agonists of Toll-like receptor (TLR) signaling activity, comprising

20   contacting a cell that expresses a TLR with a test compound and measuring a test level of TLR signaling activity,

wherein a test level that is positive is indicative of a test compound that is a TLR agonist, and

wherein the cell is a Raji cell, a RAMOS cell, a KG-1 cell, a Nalm-6 cell, a Jurkat

25   cell, a Hela cell, a Hep-2 cell, an A549 cell, a Bewo cell, an NK-92 cell or an NK-92 MI cell, and the TLR is TLR3.

174.   A screening method for identifying antagonists of Toll-like receptor (TLR) signaling activity, comprising

30   contacting a cell with a positive reference compound and measuring a reference level of TLR signaling activity,

contacting the cell with the positive reference compound and a test compound, and measuring a test level of TLR signaling activity,

wherein a test level that is less than a reference level is indicative of a test compound that is a TLR antagonist, and

wherein the cell is selected from the group consisting of a RPMI 8226 cell, a RAMOS cell, a Raji cell, a THP-1 cell, a Nalm cell and a KG-1 cell, and the TLR is TLR9.

5

175.   A screening method for identifying antagonists of Toll-like receptor (TLR) signaling activity, comprising

contacting a cell with a positive reference compound and measuring a reference level of TLR signaling activity,

10   contacting the cell with the positive reference compound and a test compound, and measuring a test level of TLR signaling activity,

wherein a test level that is less than a reference level is indicative of a test compound that is a TLR antagonist, and

wherein the cell is selected from the group consisting of a RPMI 8226 cell, a

15   RAMOS cell and a Raji cell, and the TLR is TLR7.

175.   A screening method for identifying antagonists of Toll-like receptor (TLR) signaling activity, comprising

contacting a cell with a positive reference compound and measuring a reference

20   level of TLR signaling activity,

contacting the cell with the positive reference compound and a test compound, and measuring a test level of TLR signaling activity,

wherein a test level that is less than a reference level is indicative of a test compound that is a TLR antagonist, and

25   wherein the cell is selected from the group consisting of a Raji cell, a RAMOS cell, a KG-1 cell, a Nalm-6 cell, a Jurkat cell, a Hela cell, a Hep-2 cell, an A549 cell, a Bewo cell, an NK-92 cell and an NK-92 MI cell, and the TLR is TLR3.

176.   A screening method for identifying an enhancer of a Toll-like receptor

30   (TLR) agonist, comprising

contacting a cell with a positive reference compound and measuring a reference level of TLR signaling activity, and

contacting a cell with the positive reference compound and a test compound and measuring a test level of TLR signaling activity,

wherein the positive reference compound is a TLR agonist, and a test level that is greater than the reference level is indicative of a test compound that is an enhancer of a TLR

5   agonist.

177.   The method of claim 176, wherein the positive reference compound is an immunostimulatory nucleic acid.

10   178.   The method of claim 176, wherein the positive reference compound is an imidazoquinoline compound.

180.   The method of claim 176, wherein the cell is selected from the group consisting of a KG-1 cell, a Nalm-6 cell, a Raji cell, a RAMOS cell, a Jurkat cell, a Hela cell,

15   a Hep-2 cell, an A549 cell, a Bewo cell, an NK-92 cell and an NK-92 MI cell, and the TLR is TLR3.

181.   The method of claim 176, wherein the cell is selected from the group consisting of a KG-1 cell, a Nalm-6 cell, a Raji cell, an RPMI 8226 cell, a RAMOS cell, and a

20   THP-1 cell, and the TLR is TLR9.

182.   The method of claim 176, wherein the cell is selected from the group consisting of a Raji cell, an RPMI 8226 cell and a RAMOS cell, and the TLR is TLR7.

25   183.   The method of claim 1, wherein the TLR is TLR7 or TLR9.

184.   The method of claim 172-175 or 176, wherein the cell is unmodified.
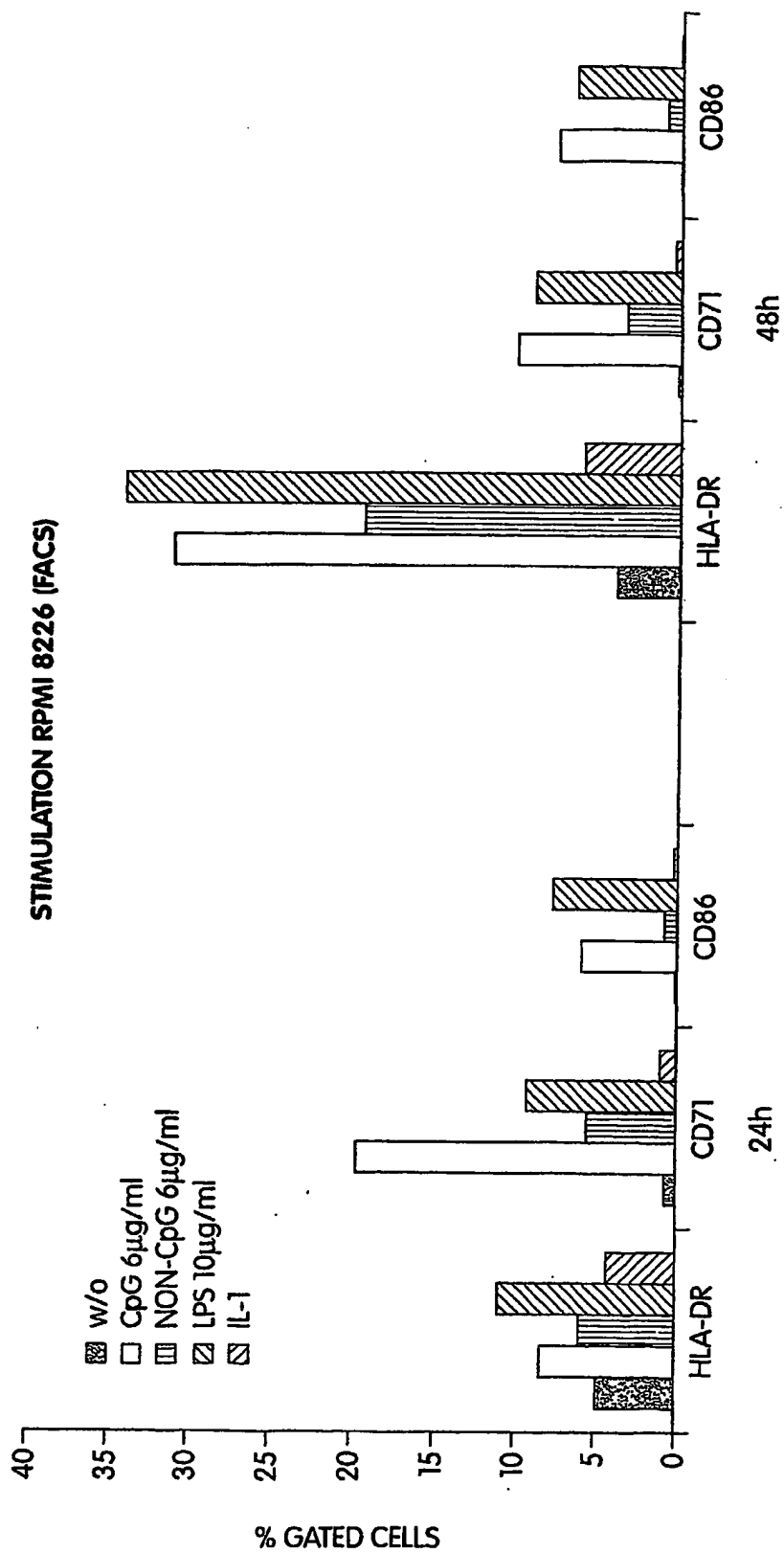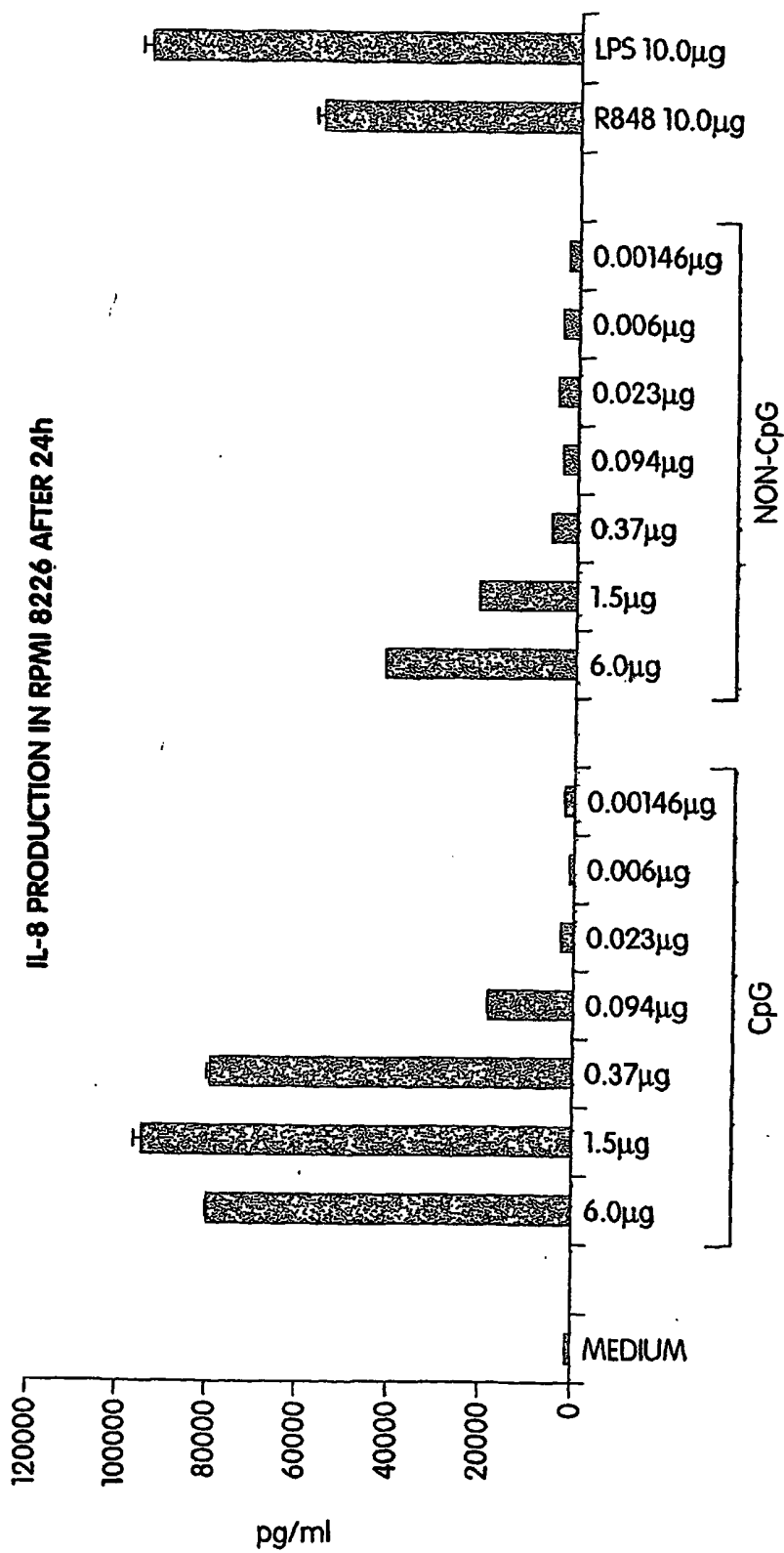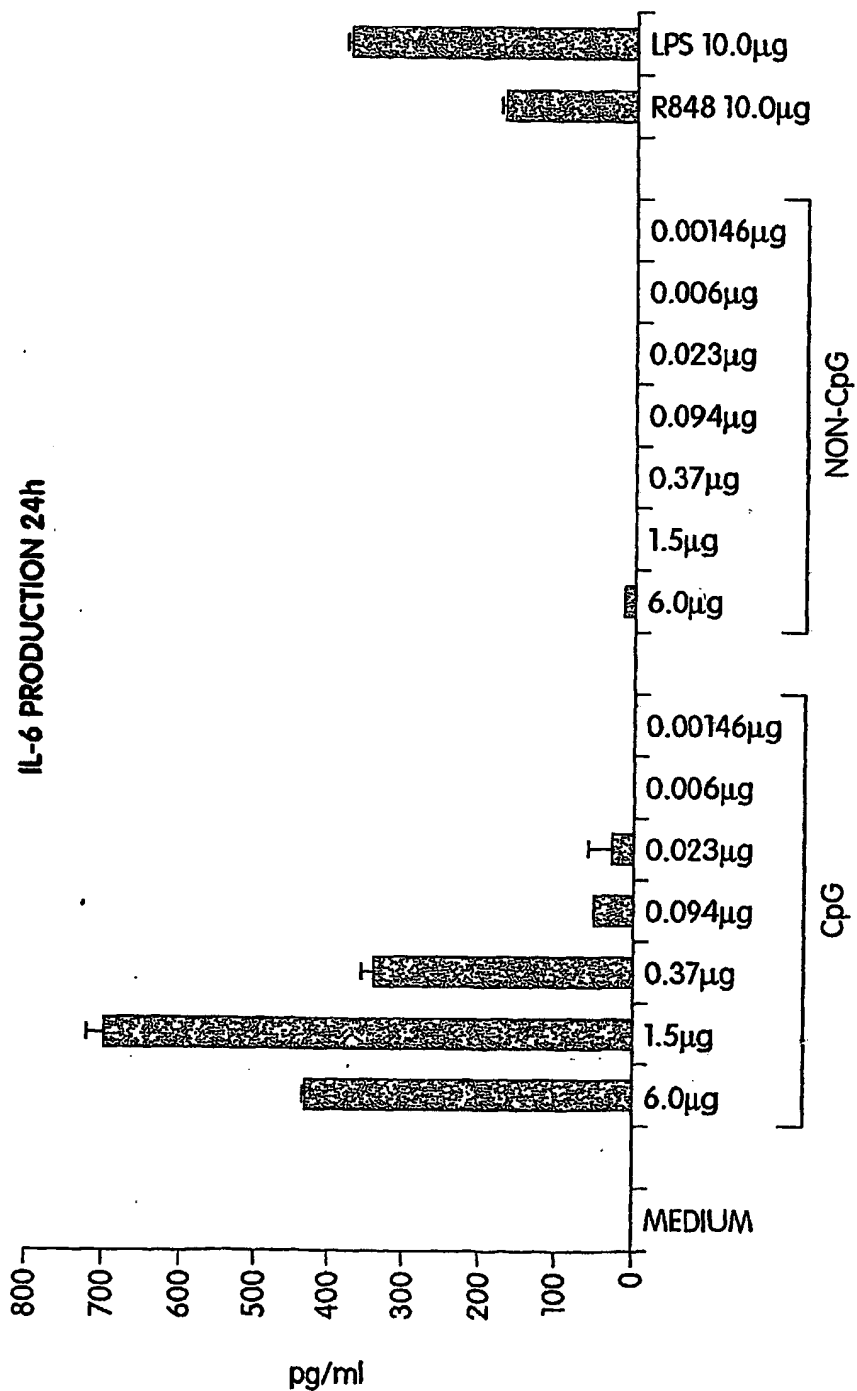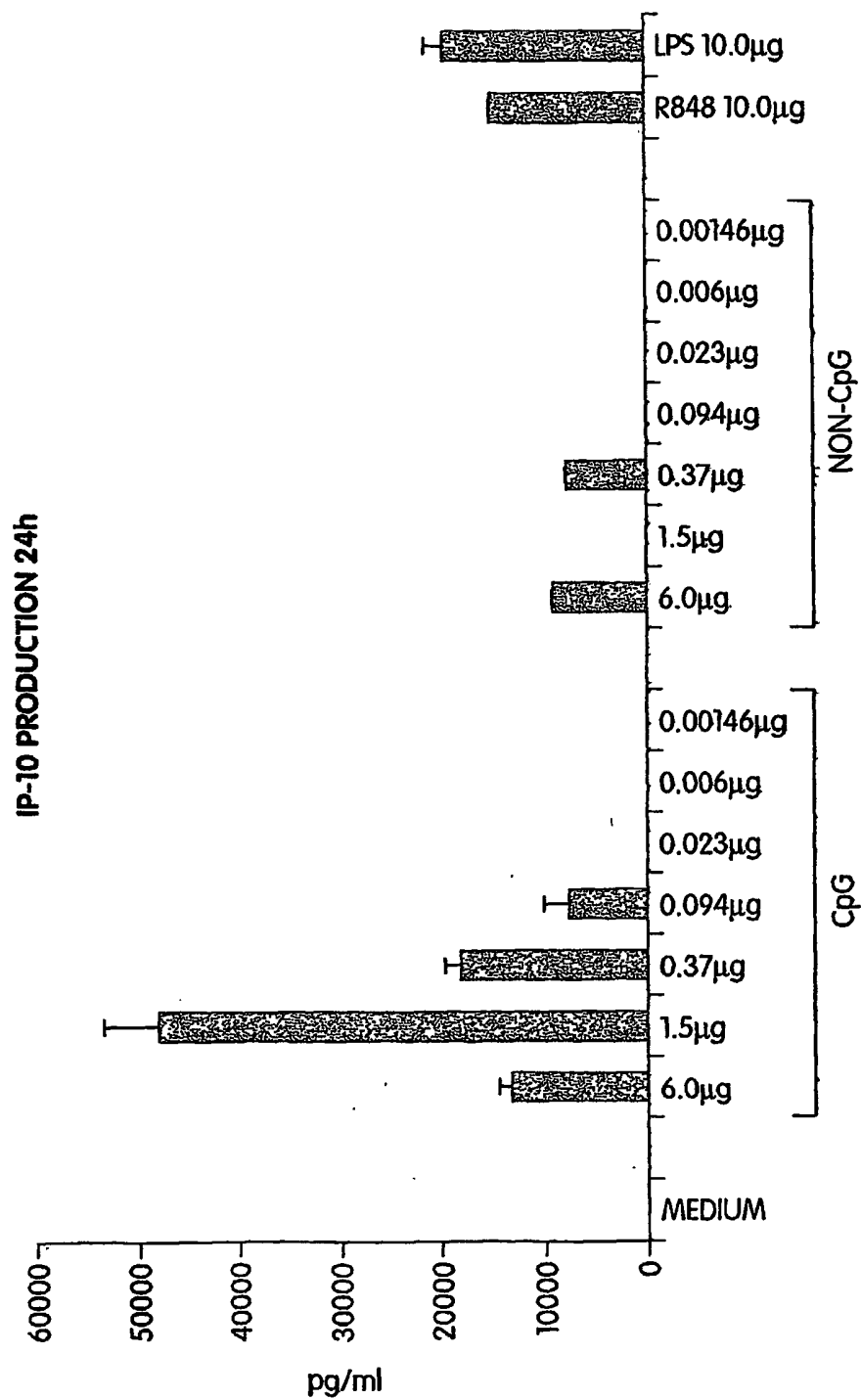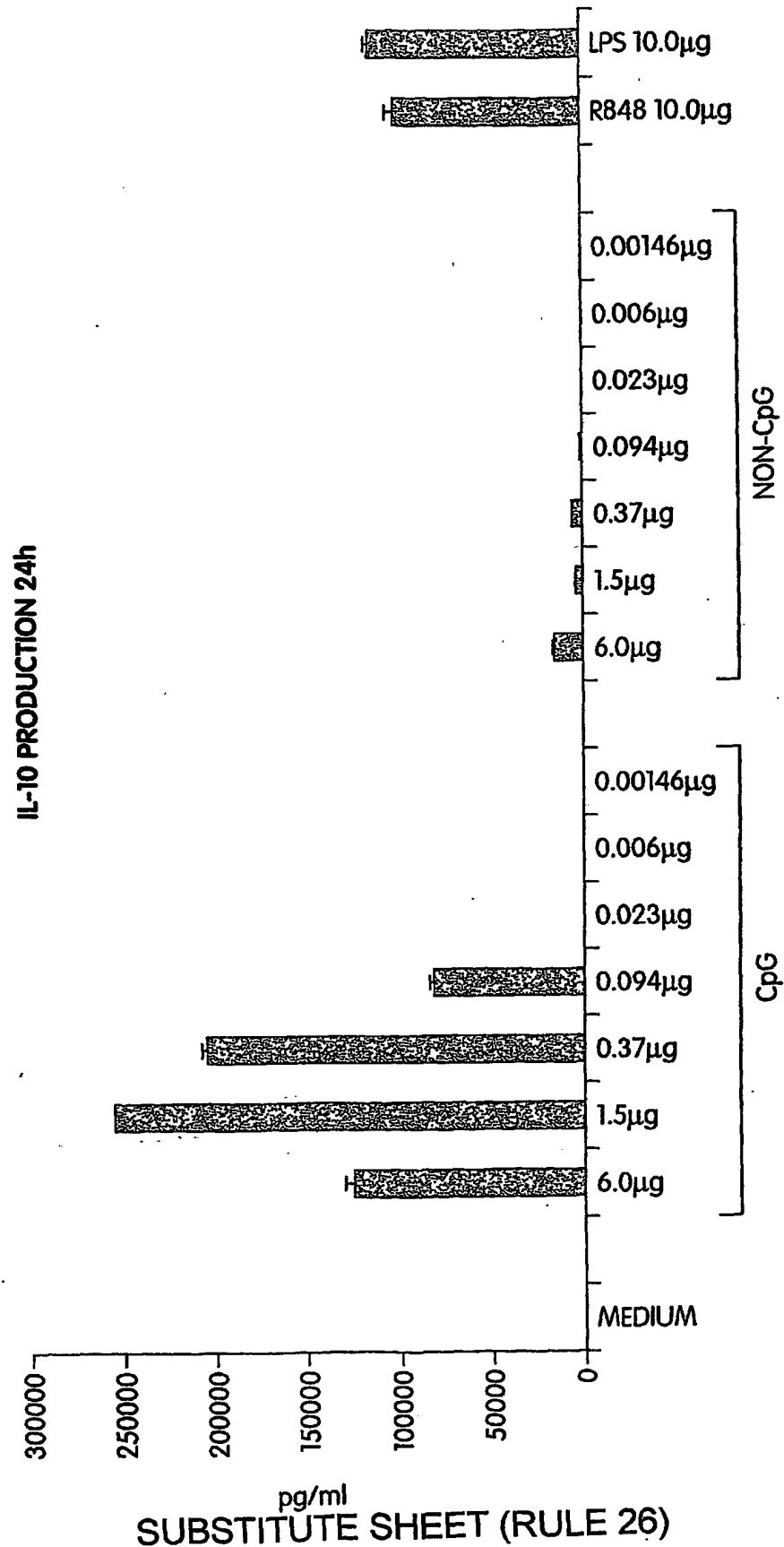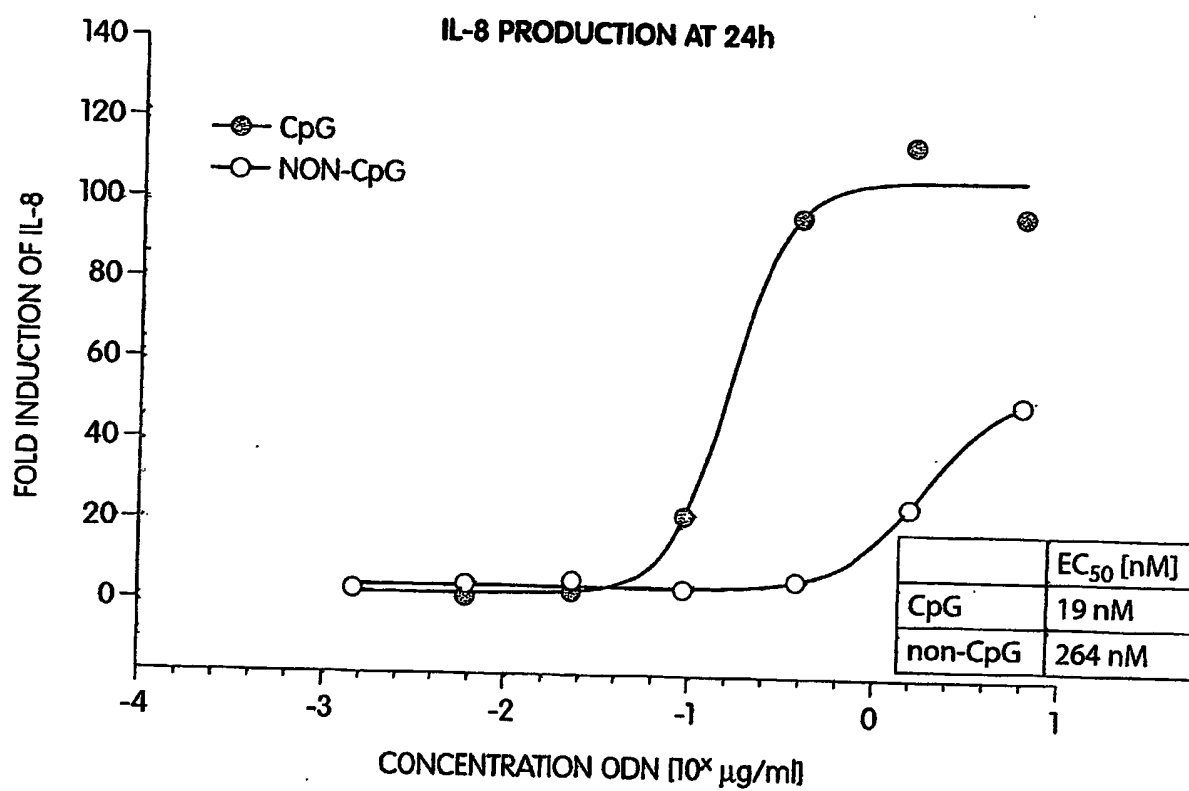
Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

IL-10 PRODUCTION 24h

Fig. 6

Fig. 7

TLR mRNA LEVELS IN RPMI 8226 CELLS

Fig. 8

Fig. 9

Fig. 10

Fig. 11

Fig. 12
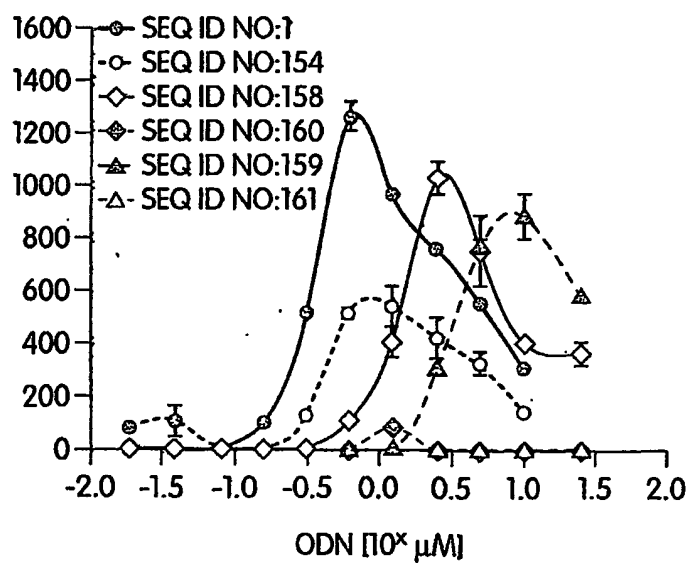
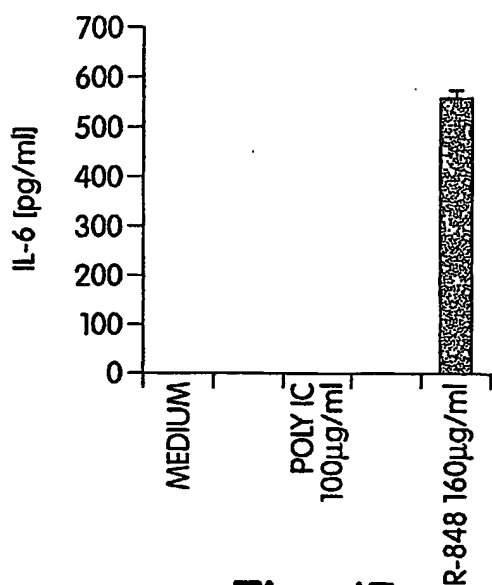**TLR EXPRESSION IN Raji CELLS**

Fig. 13

ODN [10^x μM]
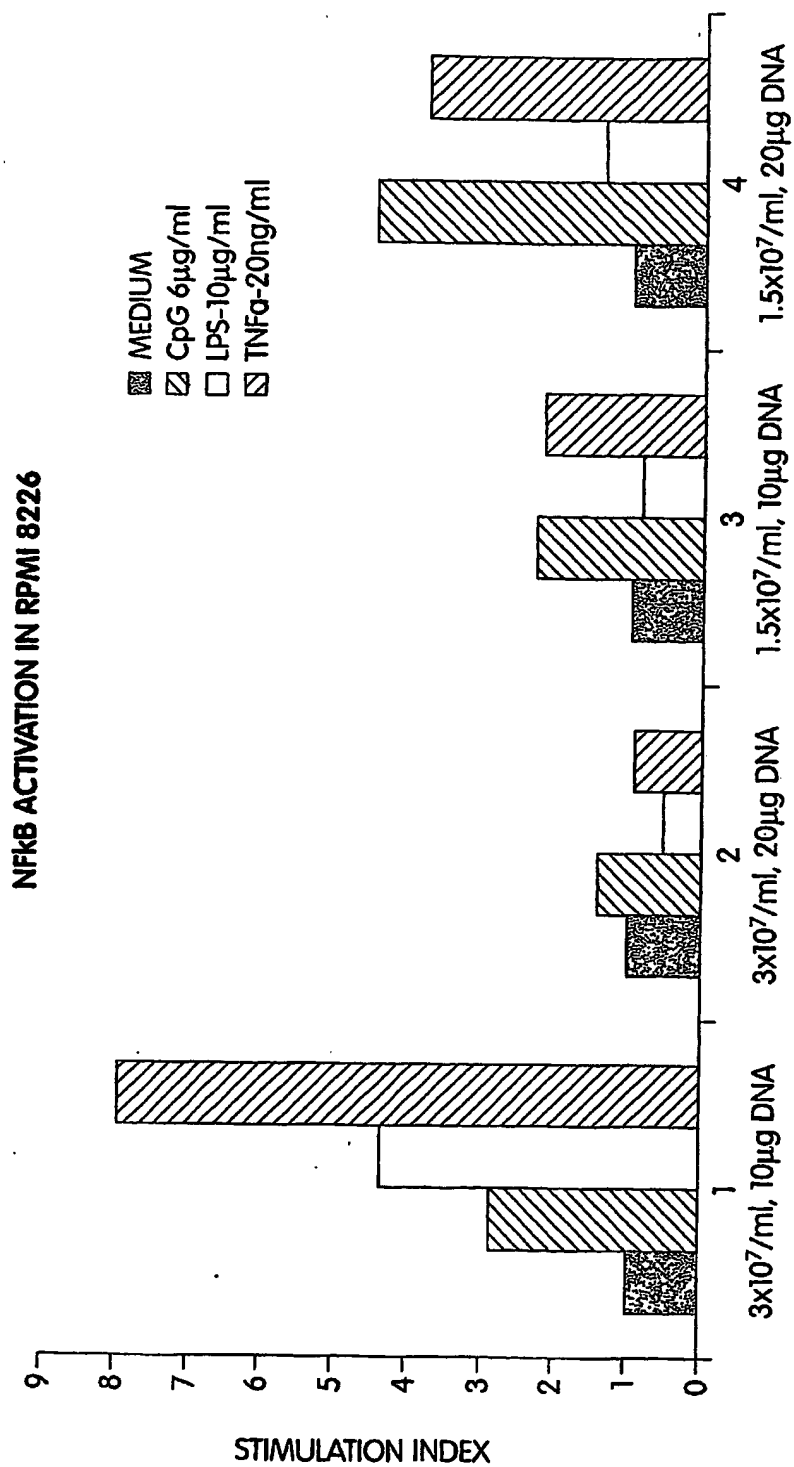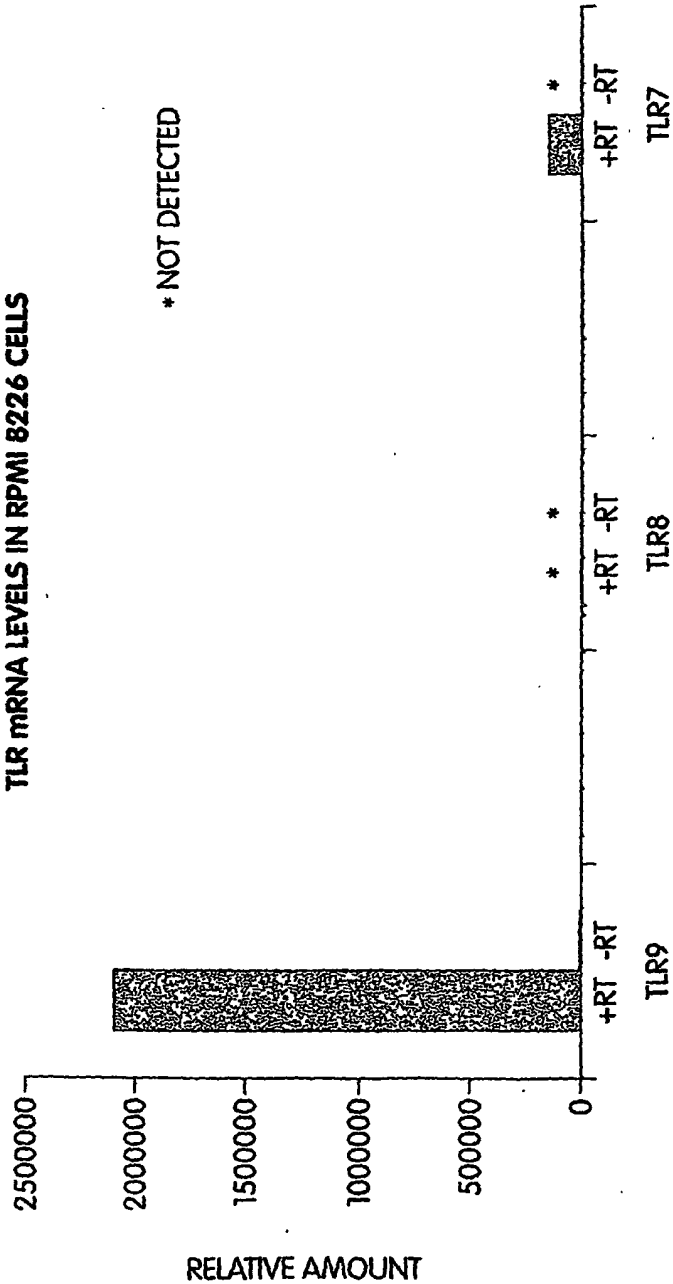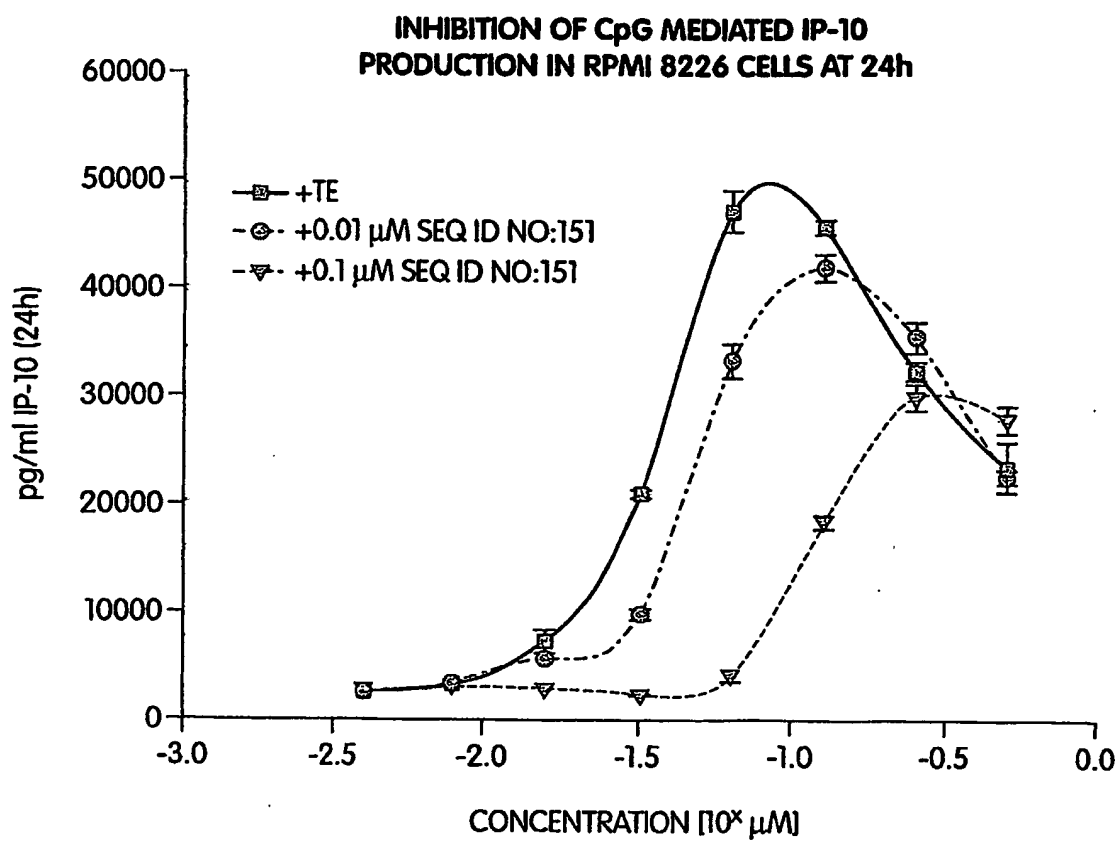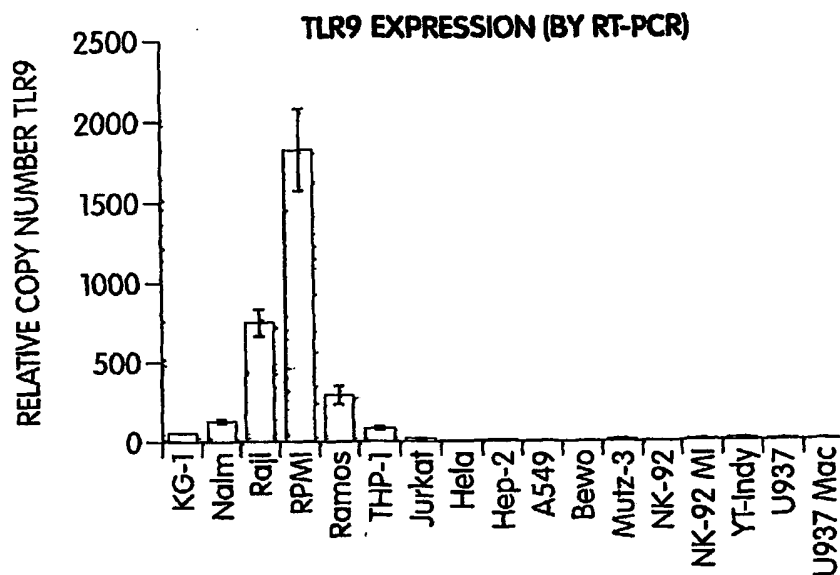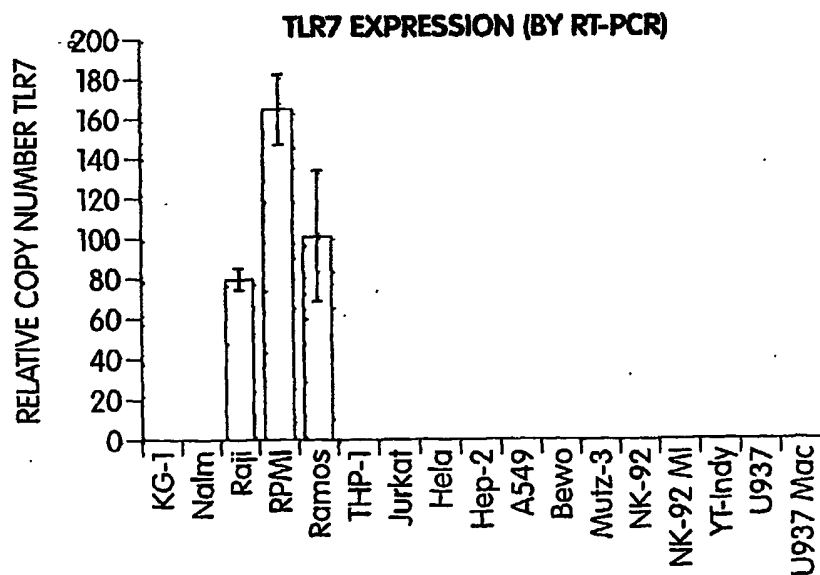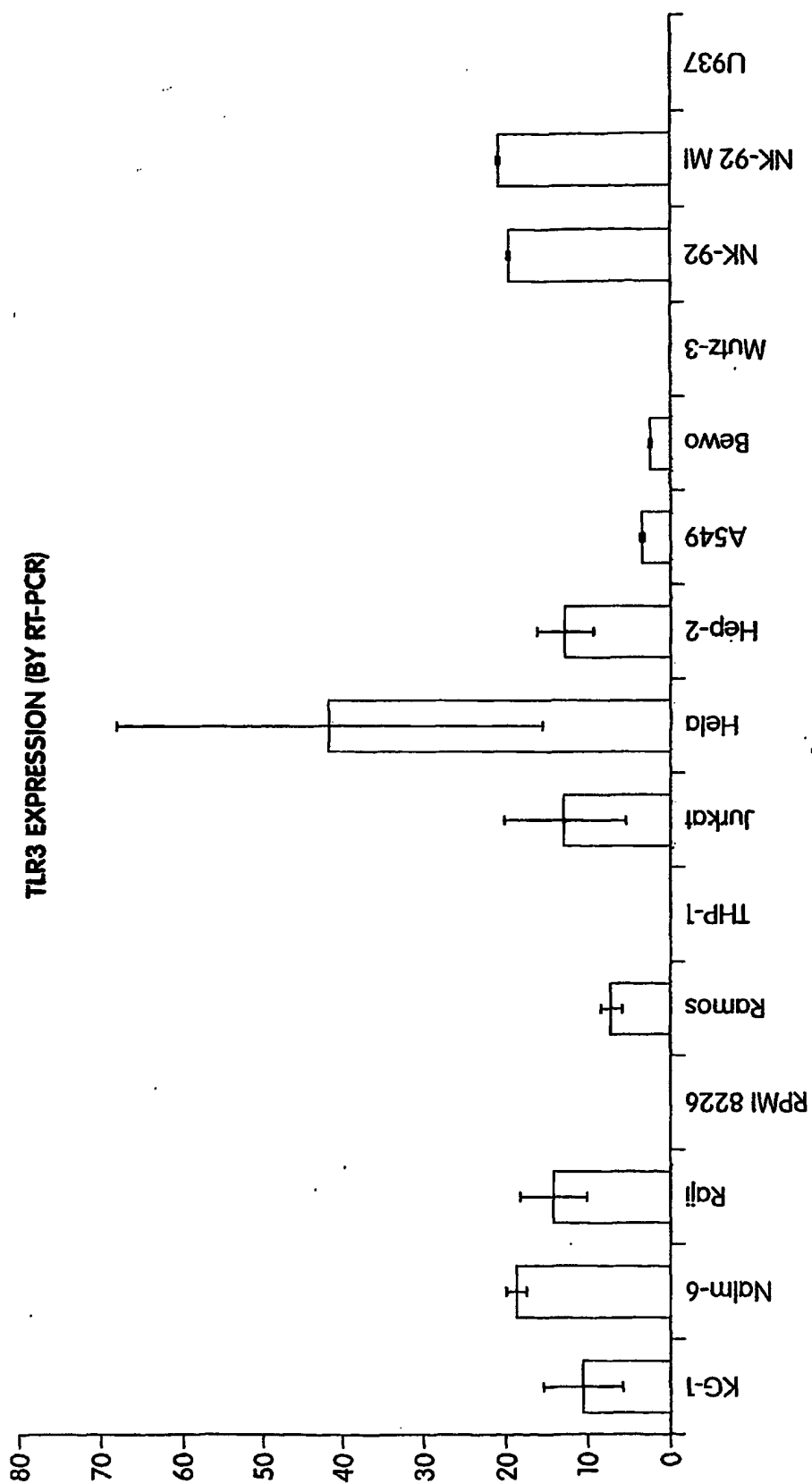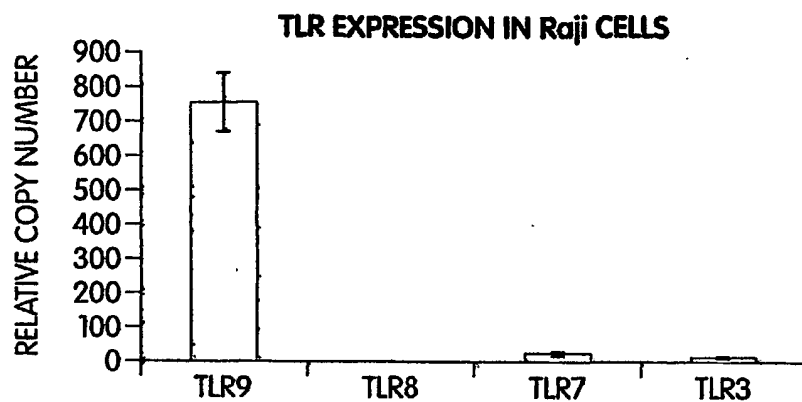
Fig. 14

Fig. 15
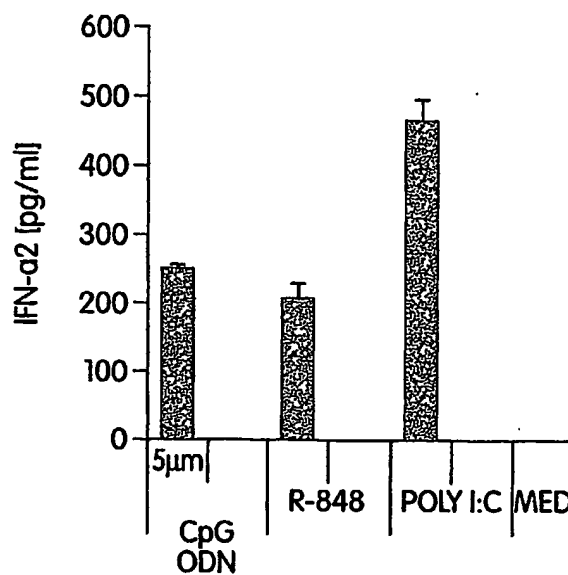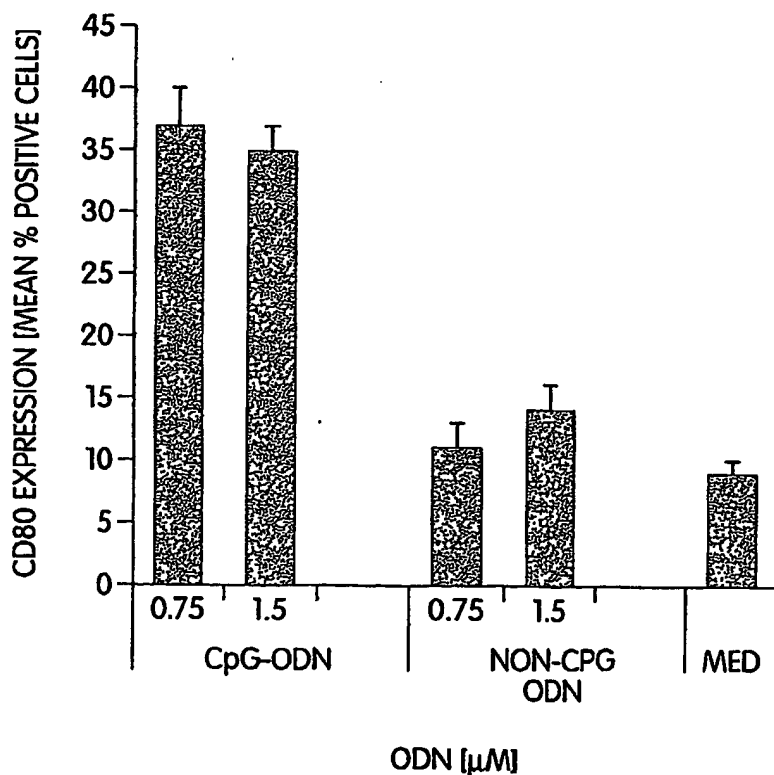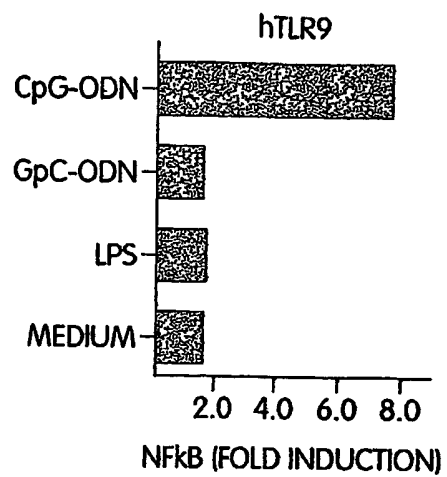

Fig. 16

Fig. 17

SUBSTITUTE SHEET (RULE 26)

Fig. 18A

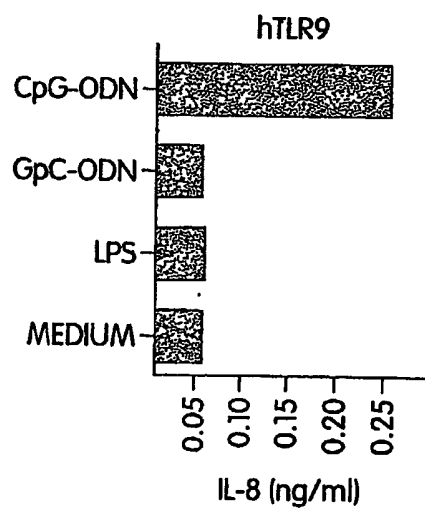Fig. 18B

293-mTLR9

NFkB-luc
(FOLD INDUCTION)

Fig. 19

293-hTLR9

NFkB-luc
(FOLD INDUCTION)

Fig. 20

Fig. 21

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